NeuroD2, ASH1, Zic1, Zic3, and MyT1, or a non-human homologous counterpart of any of these;

(B) at least one antisense oligonucleotide corresponding to the human MSX1 gene , the human HES1 gene, or a non-human homologous counterpart of either of these; and

(C) a retinoid and at least one neurotrophin from the group consisting of BDNF, CNTF, PDGF, NGF, NT-3, NT-4, and sonic hedgehog.

REMARKS

The Pending Claims

Prior to entry of the above amendments, Claims 1-27 are pending. Claims 1-4 and 26-27 are directed to a method of transdifferentiating an epidermal basal cell into a cell having one or more morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal or glial cell. Claims 5-10 are directed to a transdifferentiated cell having one or more morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal or glial cell. Claims 11 and 12 are directed to a kit for converting epidermal basal cells into cells having one or more morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal or glial cell. Claim 13 relates to a method of using transdifferentiated epidermal basal cells having one or more morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal or glial cell to isolate a novel nerve growth factor. Claim 14 relates to a method of using transdifferentiated epidermal basal cells having one or more morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal or glial cell to screen a potential new drug for treating a nervous system disorder. Claims 15-24 are directed to a transdifferentiated epidermal basal cell having one or more morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal or glial cell. Claim 25 relates to a cell culture derived from the

transdifferentiated cell of Claim 15.

The Office Action and Applicant's Amendment and Response

For the sake of clarity, Applicant has amended the specification at page 10, lines 1-15, by cleaning up the literature citations, thus making the sentence more understandable. No new matter is added by the amendment.

The amendment of Claim 1, in step (c), to recite ". . .(c) growing the transfected cell in the presence of at least one antisense oligonucleotide comprising a segment of a human MSX1 gene and/or human HES1 gene, or homologous non-human counterpart of either of these, in an amount sufficient to suppress the expression of functional MSX1 gene product and/or HES1 gene product;" is made merely for greater clarity. Support is found in the specification, for example, at page 13, lines 27-33.

The Examiner provided an initialed Information Disclosure Statement, a Notice of References Cited, and a Notice of Draftsperson's Patent Drawing Review, which indicated that the Figures were declared informal.

The Examiner acknowledged Applicant's claim for foreign priority based on an application 00101100.6, filed in the European Patent Office on January 20, 2000, and on application 2000-048291, filed in the Japanese Patent Office on January 20, 2000, but she noted that Applicant has not filed a certified copy of the foreign applications as required by 35 U.S.C. § 119(b). Applicant has ordered certified copies of the foreign applications from which priority was claimed, and Applicant anticipates that they will be available for timely forwarding to the Examiner during the pendency of the above-captioned application.

Claims 1-27 were rejected for the following reasons.

A. Rejection of Claims 1-27 under the Doctrine of Obviousness Type Double Patenting

The Examiner rejected Claims 1-27 under the Doctrine of Obviousness Type Double Patenting over Claims 1-15 of Lévesque *et al.* (U.S. Patent No. 6,087,168). The Examiner

stated that a timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. The grounds of rejection included the following:

> **Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of '428 introduce the following which are encompassed by the broad "comprising" language of the claims of '168: (1) the neurotrophins CNTF, PDGF and sonic hedgehog, and (2) the physiological and/or immunological feature is nestin, neural RNA-binding protein Musashi, neurofilament M, neural-specific beta-tubulin, neural-specific enolase, microtubule associated protein 2, glial fibrillary acidic protein (GFAP), or O4. The claims of '428 also encompass broadly any transdifferentiated cell, methods of making or using, whereby the cell is transdifferentiated into a cell having one or more morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal, or glial cell. This breadth is also encompassed by the claims of '168 due to use of the open "comprising" language in those claims.**

In response, Applicant is willing to file a terminal disclaimer in the event any claims of the above-captioned application be found allowable.

B. <u>Rejections under 35 U.S.C. § 112, second paragraph</u>

The Examiner rejected Claims 2 and 11 under 35 U.S.C. § 112, second paragraph, because Claim 2 contains a typographical error in line 3, i.e., it appears "fro" should read "from." Also, Claim 11 contains a typographical error in line 1. It appears "cells to cells into cells having" should read either "to cells" or "into cells."

In response, Applicant thanks the Examiner for pointing out these typographical errors and has amended Claims 2 and 11 to correct them.

C. <u>Rejections under 35 U.S.C. § 112, first paragraph</u>

(1) Claims 5-12 and 15-25 were rejected under 35 U.S.C. § 112, first paragraph. The Examiner based the rejection on the assertion that:

> **. . . Claims 5-12 and 15-25 are drawn to a breadth of transdifferentiated cells, kits comprising said cells and cell**

cultures having a broad scope of morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal or glial cell.

The specification as filed teaches that human adult skin was cultured and transfected with pRcCMVneo vectors containing B-gal, NeuroD1, NeuroD2, hASH1, Zicl or hMyTl human genes. The specification teaches in example 3 the design of two antisense oligonucleotides to target human MSX1 and two antisense oligonucleotides to target human HES1. In example 4, the specification teaches the methods for detection of transdifferentiation of the epidermal cells to neural cells as immunohistochemical detection of neurofilament M, neural specific tubulin, neural specific enolase, microtubule associated protein 2, neurofilaments Mix, flial fibrillary acidic protein, and morphological criteria. The specification teaches that cells with neurites longer than three cell diameters (50 microns or longer) and expressing at least one neuronal marker were counted as neurons. Table 1 teaches the results of the transdifferentiation experiments showing that a combination of neurogenic transcription factor expression coupled with decrease in MSXI and HES1 expression was most effective at establishing transdifferentiation.

The claims encompass transdifferentiated neuronal cells made by different methods or having different physiological characteristics as described above. The specification however, only teaches in Table 1 a defined set of cells having some characteristic of a differentiated neuronal cell, the structure of which is not adequately described therein. The specification teaches broadly that different characteristics of neuronal cells were evaluated, but does not specify the uniformity of such characteristics amongst or between the cells having different transcription factors and antisense sequences applied. Therefore, it is not clear to one of skill in the art that the cells taught as differentiated neuronal cells have the structures as claimed or that a representative number of such cells was described by the specification as filed . . .

Contrary to the Examiner's assertion, the specification does reasonably convey to the skilled artisan that Applicants were in possession of the claimed invention. Claims 5-10 and and Claims 15-24, are drawn to a "transdifferentiated [epidermal basal] cell having one or more morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal, or glial cell . . ." The specification discloses numerous useful examples of morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal, or glial cell and methods for detecting them. (E.g., Specification, at page 15, line 30 through 17, line 2; and at page 17, line 21 through page 18, line 22). By way of example, the specification discloses in detail how various neural-specific antigenic markers were detected on the surfaces of transfected cells (at page 27, line 22 through page 28, line 2) and that only cells that, in addition to at least one neuronal antigenic marker, expressed processes 50 microns or longer were counted as neurons (Page 28, lines 2-5).

A transdifferentiated cell in accordance with the claimed invention must have at least one morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal, or glial cell, but uniformity of antigenic markers among the individual cells is not an essential element of the claimed cell.

One skilled in the art would know that successful transfection methods typically

require a certain statistically significant number of cells. In the instant case, a transfection protocol was used in which epidermal cells were plated in 6-well tissue culture plates at a density of 50,000 cells per well. While this fact was not disclosed in the specification, one of skill in the arts of mammalian tissue culture and transfection would be familiar with such technical details and would not need to be informed of this information in order to practice the invention. The specification as filed (Figure 1C) shows a portion of a microscopic field of treated cells in monolayer– and shows more than a statistically significant number of them (greater than 30), but this is only a representative portion of the 100-300 cells in each of the five to seven fields of immuno-stained cells that were counted (e.g., at page 28, lines 28-30). Processes can be seen in a number of cells in Figure 1C, and the specification further discloses that 25% of them were neurofilament M immunoreactive (Page 8, lines 26-27).

Because for convenience the data in Table I relate to the determination of only one neural-specific antigen (neurofilament M), absolute numbers of successfully transdifferentiated cells that express another of the useful markers would be underestimated. But the relative values provided in Table I are sufficient to show one of skill in the art the relative effectiveness of various combinations of antisense oligonucleotides and neurogenic factors in accordance with the claimed method. The range of effectiveness among the various treatments illustrated in Table I extends over at least three orders of magnitude, which indicates that the values in Table I reflect real effects on as many as 26% of the treated cells, and possibly more. Thus, Table I, in conjunction with Figure 1C and other disclosures in the specification, indeed conveys that the Applicants were in possession of the claimed transdifferentiated cells.

The Examiner also stated:

> . . . Further it is not clear from the specification as filed that the cells taught as having some or several such differentiation markers could be considered differentiated neuronal cells per se. The activation of one or several genes in epidermal cells leading to the transcription of one or more neuronal markers or a single physiological response does not indicate that such modified epidermal cells would necessarily have the function of neuronal cells based on cell acquisition of one or several such morphological features.
>
> The claims drawn to kits containing ingredients for differentiation of epidermal cells to neuronal cells are further not adequately described by the specification as filed because the specification does not teach the structures of the cells

based on the application of the various kit components.

In summary, the claims are drawn to a genus of transdifferentiated neuronal cells having different characteristics yet the specification as filed does not teach the correlation of these characteristics to the methods applied for transdifferentiation of the epidermal cells. Therefore, while the cells may suggest specific neuronal features, they do not show possession of a representative number of such whole, complete, neuronal cells to show possession to one of skill in the art of the genus of differentiated neuronal cells as claimed

As noted above, amended Claims 5-12 and 15-25 are directed to a "transdifferentiated cell" or transdifferentiated epidermal basal cell" having one or more morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal, or glial cell, rather than to a neuronal cell *per se*. Thus, Applicant believes that there is no basis for the rejection on this ground.

In particular, Applicants agree with the Examiner that whether a transdifferentiated cell is a "neuronal cell per se" is not a clearly defined determination. To one of skill in the art, a "neuron" is defined by several criteria including morphology (e.g., long processes or neurites), expression of a set of neural markers (e.g., neurofilament M, neural-specific tubulin, neural-specific enolase, microtubule associated protein 2, and others), synthesis of neurotransmitters (e.g., dopamine or expression of tyrosine hydroxylase, the key enzyme in dopamine synthesis), and membrane excitability. Generally only a small operative subset of the possible criteria are examined with respect to a particular neuronal population within a given context.

It is known in the art that every neuronal population expresses a specific set of neural markers, neurotransmitters, and receptors, and that as neuronal precursor cells differentiate into other neuronal cell types in response to physiological signals in the microenvironment, the set that is expressed will be different. (E.g., see Exhibit A: D.L. Stemple and N.K. Mahanthappa, *Neural stem cells are blasting off*, Neuron 18:1-4 [1997]; Exhibit B: Y. Renoncourt *et al.*, *Neurons derived in vitro from ES cells express homeoproteins characteristic of motoneurons and interneurons*, Mechanisms of Development 79:185-97 [1998]; Exhibit C: A.J. Kalyani *et al.*, *Spinal cord neuronal precursors generate multiple neuronal phenotypes in culture*, J. Neurosci. 18(19):7856-68 [1998]; copies attached). The

transdifferentiated cells of the claimed invention are useful in that, *inter alia*, they can be manipulated, in vitro in the presence of specific exogenously supplied signal molecules, or in vivo within specific microenvironments, into diverse types as defined by the operative criteria. (E.g., see specification, at page 5, lines 3-17; at page 18, line 28 through page 19, line 7; and at page 20, lines 1-10).

As to Claims 11 and 12, directed to a kit for converting epidermal basal cells into cells having one or more morphological, physiological and/or immunological feature(s) of a neural progenitor, neuronal, or glial cell, the relationship between the components of the claimed kit and the characteristics of the claimed transdifferentiated cells are indeed described in the specification in some detail. For example, the role of the cDNA encoding a neurogenic transcription factor. . . comprising NeuroD1, NeuroD2, ASH1, Zic1, Zic3, or MyT1, is described, for example, at page 9, line 30 through page 10, line 15 (as amended); and page 12, lines 3-11. The role of the antisense oligonucleotide comprising a segment of a human MSX1 gene or HES1 gene (negative regulators of neural differentiation), or a non-human homologous counterpart is described, for example, at page 9,lines 26-29; page 10, lines 16-21; and page 13, line 21 through page 14, line 29. Induction of neural differentiation, or at least induction of neuron-like characteristics, that occurs in the presence of a retinoid and at least one neurotrophin comprising BDNF, CNTF, PDGF, NGF, NT-3, NT-4, or sonic hedgehog is described, for example at page 15, lines 13-29; page 17, lines 15-20; and page18, lines 28-32. In addition, one of skill in the art is familiar with the role of retinoids, such as retinoic acid, in inducing differentiation of some neural populations (e.g., Exhibit B: Y. Renoncourt *et al.* [1998], at 186, column 2, first complete paragraph; Section 2.3, page187, column 1 through page 189, column 2).

Therefore, Applicant respectfully requests the Examiner to withdraw the rejection of Claims 5-12 and 15-25 on this ground.

(2) Claims 1-27 were rejected under 35 U.S.C. § 112, first paragraph. While the Examiner noted that the specification is enabling for differentiated cells showing some specific neuronal cell features, Claims 1-27 were rejected for the following reasons:

> . . . Claims 1-27 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for differentiated cells showing some specific neuronal cell features, does not reasonably provide enablement for the scope of methods for making neuronal cells as claimed. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.
>
> Claims 1-27 are drawn to methods of transdifferentiating an epidermal cell into a neuronal cells and the cells produced by said process.
>
> The specification as filed teaches that human adult skin was cultured and transfected with pRcCMVneo vectors containing B-gal, NeuroD1, NeuroD2, hASH1, Zic1 or hMyT1 human genes. The specification teaches in example 3 the design of two antisense oligonucleotides to target human MSX1 and two antisense oligonucleotides to target human HES1. I In example 4, the specification teaches the methods for detection of transdifferentiation of the epidermal cells to neural cells as immunohistochemical detection of neurofilament M, neural specific tubulin, neural specific enolase, microtubule associated protein 2, neurofilaments Mix, flial fibrillary acidic protein, and morphological criteria. The specification teaches that cells with neurites longer than three cell diameters (50 microns or longer) and expressing at least one neuronal marker were counted as neurons. Table 1 teaches the results of the transdifferentiation experiments showing that a combination of neurogenic transcription factor expression coupled with decrease in MSX1 and HES1 expression was most effective at establishing transdifferentiation.
>
> Claims 1 and 5 as written broadly encompasses any differentiated neuronal cell originating as a skin cell where a DNA encoding a neurogenic transcription factor is expressed and an antisense to a negative regulator of neuronal differentiation is expressed. The specification as filed however, only teaches differentiated cells having specific physical or marker characteristics of neuronal cells as a result of specific expression of neuronal transcription factors with and without antisense suppression (see Table 1).
>
> This method of differentiation of epidermal cells to neuronal cells recites several key steps for achieving a cell having certain neuronal characteristics, but is unpredictable in the following instances:
>
> The epidermal cell obtained from the patient is not specified as to type nor are any other physical characteristics given to determine the susceptibility of the cell to dedifferentiation, further, the specification as filed does not specify how the cells were dedifferentiated . . .

Applicant disagrees that the specification lacks enablement for the claimed methods and transdifferentiated cells and cell cultures (e.g., Claim 25). For example, the specification, describes the source of the epidermal basal cells, their separation from keratinized cells, and the fact that no further dedifferentiation step is necessary (e.g., at page 11, lines 5-28). It is known that low calcium ion concentration results in the stripping of the keratin-forming upper epidermal layers from the basal cells, which is followed by rapid basal cell proliferation when calcium ion is returned to the dedifferentiated cell population. (E.g., specification, at page 11, lines 16-26); and P.K. Jensen and L. Bolund, *Low Ca^{2+} stripping of differentiating cell layers in human epidermal cultures: an invitro model of epidermal regeneration*, Experimental Cell Research 175:63-73 [1988], copy attached as Exhibit D). As the specification states, at page

11, lines 26-28, "Beyond this, it is not necessary to do a dedifferentiating step with respect to individual epidermal basal cell(s) after they are separated, isolated, or selected from the differentiated keratinized cells."

Example I demonstrates how before transfection, the [keratinized] differentiated cell layers were stripped from the basal cells in calcium-free medium and then removed from the culture (at page 25, line 21 through page 26, line 6). Further, the specification demonstrates that the claimed method of transdifferentiating an epidermal basal cell indeed works to transdifferentiate at least some large subset of the cell population (see, e.g., Examples V and VI; Table I, especially at page 30, lines 7-11), the identity of which subset is not material to enablement.

Consequently, Applicant respectfully requests the Examiner to withdraw the rejection on this ground.

The Examiner also stated:

> . . . The specification teaches only expression of certain neurogenic transcription factors in combination with certain antisense oligonucleotides resulting in cells having a phenotypic or neurogenic marker expression. Specifically example 1 teaches a calcium-free medium for growth of the skin cell culture, but does not provide guidance for what other cytokines, growth factors or genetic manipulation (claim 2) would be necessary to dedifferentiate the cells.
>
> In the case of antisense oligonucleotides to negative regulators of neuronal differentiation, there is a high level of unpredictability known in the antisense art for design of antisense molecules to known target genes (see Branch). The factors considered unpredictable are (1) delivery and stability of the antisense molecule, (2) availability of the intended target site, and (3) effective antisense action marked by a decrease in the intended target expression. The specification only teaches antisense to two genes, human MSX1 and human HES1. Therefore it would require "trial and error" experimentation to design antisense molecules to other negative regulators of neuronal differentiation as claimed.
>
> The lack of guidance in the specification as filed for these factors would therefore require "trial and error" experimentation beyond which is taught by the specification as filed. Therefore, it would require undue experimentation to practice the invention as claimed . . .
>
> . . . the antisense inhibition of the transdifferentiated cells would alone present a high level of unpredictability. There is a high level of unpredictability known in the antisense art for therapeutic, in vivo (whole organism) applications. The factors considered barriers to successful delivery of antisense delivery to the organism are: (1) penetration of the plasma membrane of the target cells to reach the target site in the cytoplasm or nucleus, (2) withstanding enzymatic degradation, and (3) the ability to find and bind the target site and simultaneously avoid non-specific binding (see Branch). Despite the synthesis of more resilient, nuclease resistant, oligonucleotide backbones and isolated successes with antisense therapy in vivo, the majority of designed antisense molecules still face the challenge of successful entry and localization to the intended target and further such that antisense and other effects can routinely be obtained. Flanagan teaches, "oligonucleotides (in vivo) are not distributed and internalized equally among organs and tissues .... Unfortunately, therapeutically important sites such as solid tumors contain very little oligonucleotide following intravenous injections in animals (page 51, column 2)."
>
> Specifically, in vitro results with one antisense molecule are not predictive of in vivo (whole organism) success. In vitro, antisense specificity to its target may be manipulated by "raising the temperature or changing the ionic strength, manipulations that are commonly used to reduce background binding in nucleic acid hybridization experiments." (Branch, p. 48) Discovery of antisense molecules with "enhanced specificity" in vivo requires further experimentation for which no guidance is taught in the specification. Note Branch who teaches the state of the art for designing an antisense which inhibits a target in vivo: it "is very difficult to predict what portions of an RNA molecule will be accessible in vivo, effective antisense

molecules must be found empirically by screening a large number of candidates for their ability to act inside cells (Branch, p.49)." And in the instant case, the therapeutic claims read broadly on administration of an antisense inhibitor in any transdifferentiated cell to a whole organism included. While the specification teaches cell culture inhibition, no evidence of successful in vivo (whole organism) antisense inhibition has been shown, nor do the culture examples correlate with whole organism delivery.

One of skill in the art would not accept on its face the successful delivery of the disclosed antisense molecules in vivo and further, treatment effects, in view of the lack of guidance in the specification and the unpredictability in the art. Neither the specification nor technology today teach general guidelines for successful delivery or treatment effects of antisense molecules in whole organisms. Specifically the specification does not teach (1) stability of the antisense molecule in vivo, (2)effective delivery to the whole organism and specificity to the target tissues, (3) dosage and toxicity, nor (4) entry of molecule into cell and effective action therein marked by visualization of the desired treatment effects. These key factors are those found to be highly unpredictable in the art as discussed supra. The lack of guidance in the specification as filed for these factors would therefore require "trial and error" experimentation beyond which is taught by the specification as filed.

Therefore, it would require undue experimentation to practice the invention as claimed . . .

Contrary to the Examiner's assertion that ". . . it would require 'trial and error' experimentation to design antisense molecules to other negative regulators of neuronal differentiation as claimed," no antisense oligonucleotides targeting *other* negative regulators are claimed in the method of Claim 1. Applicant believes that Claim 1, even before the present amendment did *not* imply that antisense oligonucleotides directed to suppressing negative regulators *other* than MSX1 and HES1 are used in the method, since only MSX1- and HES1-targeting antisense oligonucleotides were recited in step (c) of Claim 1, as originally filed. Nevertheless, merely for the sake of greater clarity, Applicant has amended Claim 1, in step (c), to recite ". . .(c) growing the transfected cell in the presence of at least one antisense oligonucleotide comprising a segment of a human MSX1 gene and/or human HES1 gene, or homologous non-human counterpart of either of these, in an amount sufficient to suppress the expression of functional *MSX1 gene product and/or HES1 gene product* . . ."

In addition, the Examiner's remarks, and the cited Flanagan *et al.* and Branch references, regarding the unpredictability of antisense therapy *in vivo* are irrelevant and clearly not properly directed to the claims as filed, which relate to the use of *in vitro* transfection methods (e.g., Claim 1, step [b]; Claim 13, step [a]; and Claim 14, step [a]). The claimed invention does not relate to in vivo delivery of antisense molecules or any other variety of in vivo gene therapy.

Consequently, Applicant respectfully requests the Examiner to withdraw the rejection

on this ground.

The Examiner further stated:

> . . . In regards to the screening and diagnostic claims, the specification as filed teaches only prophetically methods of application for screening and therapeutic applications of the transdifferentiated cells of claims 1 or 5.
>
> The specification as filed therefore does not provide any guidance for the transplantation of transdifferentiated cells/tissues into a patient nor does it provide any evidence of the cells ability to form functional connections and operate as true neurons either in vitro or in a whole organism.
>
> The factors considered unpredictable for such treatment would include the ability of the cells to retain the transdifferentiated state, for example. . .
>
> . . . Further, no guidance is taught by way of example for assaying the effect of the potential new drugs on a physiological or molecular biological property of said transdifferentiated neuronal cells. The only physiological or molecular biological properties taught are those characteristics applied to determination of the neuronal status of the transdifferentiated cells such as morphological occurance of a neurite and immunological expression of neuronal antigens.

In order to meet her burden in rejecting the claims as lacking enablement, the Examiner is required to provide a reasonable basis to question the enablement provided for the claimed invention. (*In re Wright*, 999 F.2d 1557, 27 USPQ2d 1510 [Fed. Cir. 1993]). This burden includes providing sufficient reasons for doubting any assertions in the specification as to the scope of the enablement. Only if the Examiner meets her burden does the burden shift to Applicant to provide suitable proofs indicating that the specification is enabling. In addition, an Applicant need not have actually reduced the invention to practice prior to filing. (*Gould v. Quigg*, 822 F.2d 1074, 1078 [Fed. Cir. 1987]). Prophetic examples based on predicted results are allowed for purposes of compliance with the requirements of 35 U.S.C. § 112, first paragraph. (MPEP 2164.02).

The Examiner's assertion, that the specifications provide no guidance for the transplantation of transdifferentiated cells/tissues into a patient nor evidence for the cells' ability to form functional connections and operate as true neurons . . . in a *whole organism*, is irrelevant to the claimed *in vitro* methods of using cells transdifferentiated from epidermal basal cells to isolate a novel nerve growth factor (e.g., Claim 13) or to screen a potential chemotherapeutic agent to treat a nervous system disorder (e.g., Claim 14). The claimed invention does not relate to transplanted cells.

As to whether the cells' ability to form functional connections and operate as true

neurons in vitro, this also does not bear on the enablement provided by the specification, as stated in the specification, e.g., at page 24, lines 19-27. In accordance with the methods of Claim 13 and 14, the transdifferentiated cells are assayed in vitro to determine whether there is an effect of a potential nerve growth factor or chemotherapeutic agent on a physiological or molecular biological property of the transdifferentiated cells. The use of transdifferentiated epidermal basal cells bypasses the difficulties in isolating and culturing neuronal cell types from the brain or human fetal tissue. (See, specification, e.g., at page 8, lines 17-19; and at page 23, lines 6-8). As noted above, the specification teaches a method of transdifferentiation of epidermal basal cells and discloses that the further course of development of the transdifferentiated cells depends on the in situ environmental cues to which they are exposed. Thus, the transdifferentiated cells can be manipulated to express a set of properties (e.g., morphological or antigenic) that is characteristic of certain populations of neurons. The transdifferentiated cells may or may not express all the biochemical, morphological, physiological and functional characteristics of a given neuronal or glial cell population. For example, they may or may not form functional interneuronal connections. But regardless, they are at least useful simulations of neurons or glial cells for screening or isolating promising new drugs or neural growth factors. Once the potential of a chemical agent is identified by the claimed methods, then, of course, further research can be done to verify its actual effect on particular cell populations of the nervous system and ascertain its clinical usefulness. (See, specification, e.g., at page 23, lines 1-5; and especially at page 24, lines 19-27). Therefore, the methods claimed in Claims 13 and 14 are enabled, regardless of whether interneuronal connections are formed in vitro.

Also contrary to the Examiner's assertion, the specification as originally filed indeed provides guidance for assaying the effect of the potential new drugs (e.g., Claim 14), or novel nerve growth factors (e.g., Claim 13), on a physiological or molecular biological property of the transdifferentiated neuronal cells, other than the morphological occurrence of a neurite and immunological expression of neuronal antigens. As can be seen (e.g., specification, at page

22, line 25 through page 24, line 27), the useful assay techniques described for detecting effects of potential chemotherapeutic agents [or novel growth factors] on particular cell types include those based on electrophysiological characteristics (e.g., patch clamp or intracellular electrophysiological recording [e.g., page 23, lines 21-22]), gene expression profiles, neurotransmitter profiles, cytoskeletal organization, organization of ion channels and receptors, and effects on cell survival (e.g., page 23, lines 23-25 and page 23, line 30 through page 24, line 2), which are detectable by methods known to those skilled in the arts of molecular biology and cell culture.

The Examiner has failed to meet the burden of providing sufficient reasons to doubt the assertions in the specification. Therefore, Applicants respectfully request that the Examiner withdraw the rejection of Claims 1-27 on this ground.

CONCLUSION

In view of the above amendments and remarks, it is submitted that this application is now ready for allowance. Early notice to that effect is solicited. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney at (213) 896-6665.

Respectfully submitted,
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Minireview

Neural Stem Cells Are Blasting Off

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Introduction

Over the past 5 years, there has been a flurry of excitement over the identification of putative neural stem cells. Why all the fuss? Stem cells, as the name implies, are cells from which other cell types arise and thus are of great interest to developmental biologists. The most general type of stem cell is the fertilized egg; from this single cell arise the myriad cell types that constitute a mature multicellular animal. Operationally, however, the appellation is given to cells with far less developmental potential; in a given tissue, one can speak of stem cells that have the ability to give rise to all of the differentiated cell types associated with that specific tissue. Several properties of these cells provide a useful operational definition. Specifically, stem cells are undifferentiated cells capable of proliferation, self-renewal, and "asymmetric" division. The latter allows for self-renewal (i.e., the production of new stem cells) as well as the production of differentiated progeny. All tissues contain tissue-specific stem cells during organogenesis, as do adult tissues capable of regeneration. In such tissues, latent stem cell populations are sparked into action by stress, injury, etc. (reviewed by Potten and Loeffler, 1990).

What then is a neural stem cell? In a strict sense, it should be a cell that gives rise to a variety of neurons and glia. Identification of such cells in the embryonic nervous system is a recent feat, but given the seeming lack of neural regeneration in the adult mammalian central nervous system (CNS), the idea that latent stem cells exist here, too, has seemed heretical. While this view is now in flux, current discussions of neural stem cells are characterized by ambiguous vocabulary and a plethora of disparate observations. A resolution of this muddle is not merely of academic interest. An understanding of neural stem cell biology will have profound consequences for the treatment of neurological diseases through ex vivo manipulation of such cells for transplantation or in vivo activation of quiescent neural stem cells to promote healing from within. In this minireview, we will address several issues. First, what is the evidence for the existence of neural stem cells in mammals? Second, how do regional differences in the nervous system affect neural stem cell behavior? Finally, what are the similarities among reported neural stem cells?

Central to our definition of stem cells is the property of self-renewal. The most direct method to assess the self-renewal of putative stem cells is to mitotically expand candidate cells in isolation. By subcloning the resultant progeny, one can examine the founder cell's ability to produce differentiated cell types and additional stem cells. Cells capable of proliferation and neurogenesis in such a paradigm, but which fail to self-renew, are best termed progenitor cells rather than stem cells. Detailed analyses demonstrating self-renewal have been accomplished only within a few neural structures: the neural crest, the embryonic cerebral cortex, and the embryonic striatum.

Peripheral Neural Stem Cells

Clonal primary cultures of rat neural crest have revealed that single cells that coexpress nestin and low affinity nerve growth factor receptor can produce large colonies containing neurons, Schwann cells, and smooth muscle cells (Stemple and Anderson, 1992; Shah et al., 1996). Subcloning experiments showed that these cells self-renew, and hence these cells were designated neural crest stem cells (NCSCs). The fact that these cells can be grown in clonal culture has allowed the direct assessment of the activity of purified growth factors. In recent experiments, NCSCs were found to differentiate in response to several different growth factors along different pathways (Shah et al., 1994; 1996). In response to neuregulin, nearly 80% of NCSCs respond to form only Schwann cells. By contrast, neurogenesis is induced by bone morphogenic proteins 2 and 4 (BMP2/4), and smooth muscle cells are generated in response to transforming growth factor β1. It appears that these growth factors act directly to convert NCSC into differentiated progeny. This is most apparent with BMP2/4 treatment. Within 6 hr of such treatment, well before overt neuronal differentiation, NCSCs express MASH1, a transcription factor that is critical for the differentiation of certain neuron subtypes. Furthermore, this treatment results in the complete loss of stem cells, i.e., treated cells lose their capacity to self-renew. Hence, the ability of NCSCs to self-renew can be overridden by environmental signals, and this may explain the paucity of such cells in the adult.

It is interesting to note that there is a site of peripheral neurogenesis that is likely to host stem cells throughout adulthood, the olfactory epithelium (OE). The OE is a site in which neurogenesis occurs throughout the lifetime of all vertebrates. While it is possible to culture OE-derived cells and demonstrate in vitro neurogenesis (Calof and Chikaraishi, 1989; Pixley, 1992; Mahanthappa and Schwarting, 1993), it has been difficult to identify a cell that qualifies as the olfactory stem cell. It is thought that the putative stem cell resides in the basal OE, but identification of such a cell is a topic of current research. Nevertheless, it is noteworthy that mice in which expression of the MASH1 gene has been deleted, only show severe deficits in numbers of sympathetic ganglion neurons (an NCSC derivative) and OE neurons (Guillemot et al., 1993). Thus, neural stem cells in the periphery may share common properties.

Central Neural Stem Cells

In the cerebral cortex, self-renewal has been directly demonstrated for a multipotent stem cell isolated from embryonic rat. Using medium conditioned by astrocytes and meningeal cells, Davis and Temple found that about

[illegible]% of clones produce [illegible]dreds of cells and divided for weeks (1994). About 40% of these large clones produced neurons, oligodendrocytes, and astrocytes. Self-renewal of the stem cell was demonstrated by the subcloning of several clones. For each of these clones, at least one subclone was of the large, multipotent type. This system should allow a detailed examination of the relationship between cortical stem cells and their differentiated progeny, and the identification of factors controlling differentiation decisions. In particular, this system will be useful to establish the relationship between neuronal and glial lineages.

Under different culture conditions, Reynolds and Weiss have identified a CNS stem cell from the embryonic striatum. In low density culture of embryonic striatum, a subset of cells were found to proliferate when grown in suspension in the presence of epidermal growth factor (EGF) (Reynolds et al., 1992). These cells are reported to form clonal spheres of nestin expressing cells, few of which express markers of differentiation. When the spheres are dissociated and grown on an adherent substrate in the absence of EGF, they differentiate into mixed colonies of neurons, astrocytes and oligodendrocytes. Additional studies report that these spheres can be dissociated to generate clonal secondary spheres that retain the ability to produce neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1996).

The subependymal zone adjacent to the striatum is found to be a source of an adult version of the EGF responsive multipotential CNS stem cell (Reynolds and Weiss, 1992; Morshead et al., 1994). As with the embryonic form, the EGF-responsive adult stem cell expresses nestin and undergoes extensive proliferation in suspension cultures to form large spheres that differentiate when plated on an adherent substrate. In a similar study, basic fibroblast growth factor (bFGF) was also found to stimulate sphere formation in cultures of adult striatal subependyma (Gritti et al., 1996). Importantly, in the latter study, a clonal analysis of bFGF-stimulated spheres demonstrated that these cells can self-renew and generate neurons, oligodendrocytes, and astrocytes.

It is difficult to know whether NCSCs, cortical stem cells, and striatal stem cells are distinct cell types, given the fact that they have been identified using such different culture systems. Identity among these cells seems unlikely; for example, NCSCs produce neurons in response to BMPs 2 and 4, while embryonic striatal stem cells produce astrocytes in response to a variety of BMPs including BMP2 and 4 (Gross et al., 1996). Nonetheless, it would be informative to compare the three cell types directly under identical culture conditions.

Progenitors

A number of reports detail the properties of a variety of neural progenitors. One of the most extensively studied neural progenitors, oligodendrocyte-type II astrocyte (O-2A) progenitor, is the most likely source of oligodendrocytes throughout the CNS. Originally isolated from embryonic rat optic nerve, the O-2A progenitor produces both oligodendrocytes, marked in culture by morphology and the expression of galactocerebroside (Gal-C), and type II astrocytes marked by expression of the intermediate filament, glial fibrilary acidic protein [illegible]AP) (reviewed by B[illegible]res and [illegible], 1994; R[illegible] 1989). The choice of cell fate was found to be controlled by a number of growth factors. For example, the combination of platelet derived growth factor (PDGF) and bFGF allows the extended proliferation of undifferentiated progenitors. Ciliary neurotrophic factor (CNTF) was found to induce type II astrocyte differentiation in the presence of extracellular matrix. Pro-oligodendrocytes, expressing O4 glycoside but not Gal-C, are generated in response to PDGF. Recently, neuregulin has also been shown to act as a mitogen and to prevent differentiation of the O-2A progenitor and its derivatives (Canoll et al., 1996). Given these observations, the O-2A progenitor may well be a stem cell, albeit of more restricted differentiation potential than the above-described stem cells. Nevertheless, such a designation will require formal clonal analysis that includes subcloning to demonstrate self-renewal. Further, the significance of the type II astrocyte has been called into question since attempts to identify an in vivo counterpart have failed (Williams et al., 1991), and histological and cell culture evidence support the view that astrocytes can derive from radial glia (Culican et al., 1990). Thus the O-2A counterpart in vivo may generate solely oligodendrocytes.

The study of Kilpatrick and Bartlett (1993) demonstrated that large multipotent clones can be obtained from embryonic mouse telencephalon/mesencephalon cells when grown in the presence of serum and bFGF on an adherent substrate. Although this study did not directly demonstrate self-renewal, >40% of the clones contained 3000–4000 cells by 10 days in culture. One quarter of all clones contained neurons and more than half of the neuron-containing clones also contained astrocytes. Interestingly, mature oligodendrocytes were never found to develop in these cultures.

Regional Identity and Stem Cell Behavior

Stem cells provide one mechanism for the generation of cellular diversity. For example, within a given region of the brain, a stem cell can generate a variety of cell types. In addition, neurons from different regions of the brain can have distinct region-specific properties. One major question in stem cell biology concerns the developmental potential of a stem cell. Several studies directly address the issue of developmental potential of isolated neural progenitors through heterotopic transplantation.

Recently, insightful experiments have been performed using adult hippocampal progenitors (AHPs). Cells isolated from adult hippocampus were found to proliferate in response to bFGF in a chemically defined medium. Under these culture conditions, the hippocampal cells were found to survive, and varying percentages express neuronal or glial markers. Strikingly, it is reported that these cells can be maintained in culture through multiple passages for 1 year, and when transplanted integrate and generate mature granule cell neurons in host hippocampus without forming tumors (Gage et al., 1995).

Depending on the site of transplantation, AHPs demonstrate unforeseen plasticity (Suhonen et al., 1996). When these cells were grafted into the rostral migratory pathway, the normal source of olfactory bulb (OB) granule cells, they generated mature OB neurons. Some of the grafted neurons expressed tyrosine hydroxylase

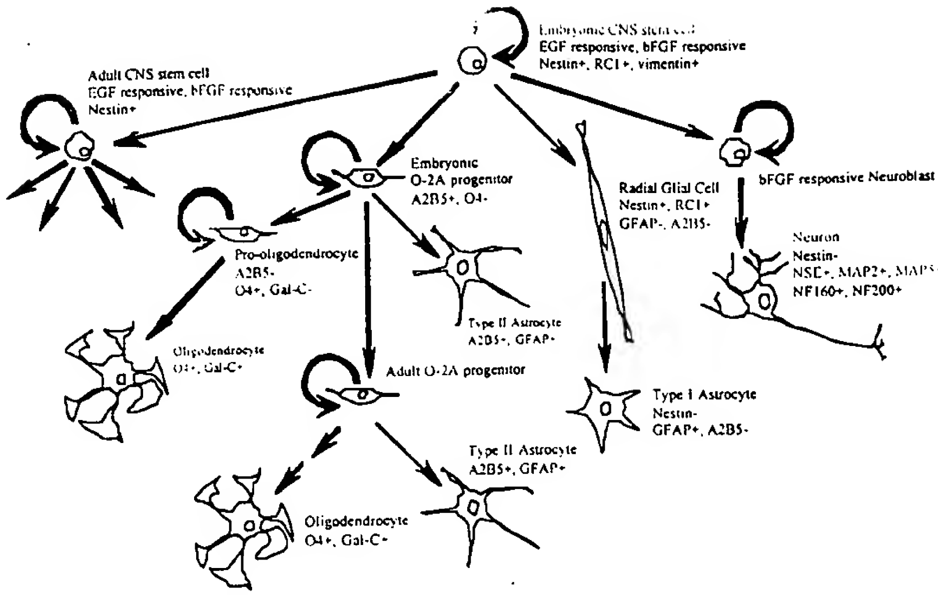

Figure 1. Idealized Lineal Relationships between the Major CNS Cell Types

In an effort to unify the studies of neural stem cells, we offer this diagram depicting the relationships between the cell types discussed. At the top is the embryonic CNS stem cell, which can self-renew and produce differentiated progeny, perhaps by a gradual restriction mechanism where intermediate cell types such as the O-2A progenitor or neuroblasts serve as the immediate precursors of the terminally differentiated cell types.

(TH), a neurotransmitter biosynthetic enzyme not normally expressed by hippocampal neurons. When the same cells were transplanted to the cerebellum, however, they failed to generate neurons though they survived at rates comparable to hippocampus or RMP grafts. Interestingly, GFAP⁻ cells were produced by the AHPs in all three sites.

Self-renewal has not been formally demonstrated in clones of AHPs nor has the relationship between AHP-derived neurons and glia been established. It would be interesting to know if single AHP cells can form neurons and glia. Alternatively, AHP cells may be of two types that proliferate in bFGF–one that is essentially a neuroblast capable only of generating neurons, and one that is a glioblast that proliferates simultaneously with the neuroblasts. Given the fact that AHPs and adult striatal progenitors show a similar response to bFGF (Gritti et al., 1996), these cells may represent essentially the same stem cell subject to slightly different culture conditions. A direct comparison of clones derived from the two populations under identical culture conditions would clarify the relationship between the two adult progenitor cell types.

While AHP cells failed to adopt cerebellar neuron identities, progenitors of cerebellar origin can adopt hippocampal identities. The external germinal layer (EGL), which normally serves as the source of cerebellar granule cell progenitors, is an actively proliferating region through the first few weeks of postnatal life in rodents. Labeled cells taken from newborn rats or transgenic mice have been transplanted into the dentate gyrus of newborn rats. When host animals were analyzed, hippocampal granule cell neurons were found to develop from transplanted progenitors. Importantly, transplant-derived hippocampal neurons were found to express calbindin and to express c-fos in response to kainic acid treatment. Normal cerebellar granule cells do not express calbindin or display the kainic acid response. Hence, metencephalic progenitors were found capable of adopting telencephalic fates when subjected to the environment of the dentate gyrus (Vicario-Abejon et al., 1995).

Can these two studies be reconciled? It is important to note that the EGL only exists for a few weeks after birth. In the study of Suhonen et al. (1996), AHPs were grafted to the adult (>3 month old) cerebellum, after granule cell neurogenesis had taken place. Therefore, it may not be particularly surprising that the cues necessary to signal cerebellar differentiation are no longer present. Thus, it would be informative to graft AHPs into the newborn cerebellum to examine whether AHP plasticity is contingent on the persistence of appropriate environmental cues. Interestingly, immortalized EGL cell lines transplanted into developing cerebellum give rise to granule and basket cell neurons (Snyder et al., 1992), and when transplanted into the lateral ventricles of newborn mice, display an ability to populate regions of the brain as diverse as the cerebral cortex (Snyder et al., 1995). Immortalization may somewhat alter the regional specificity of the cells since untransformed EGL progenitors apparently produce only granule cell neurons. Nevertheless, it is possible that some progenitor cells of cerebellar origin show greater developmental potential than those of the hippocampus.

Unifying Principles?

Here, we have summarized just a fraction of the many recent studies concerning mammalian neural progenitors. Taken together, these studies provide convincing evidence for at least one type of neural stem cell for the CNS and PNS, respectively. There may be, however,

several different CNS and PNS stem cell types. Differences in cell culture conditions, regional sources of material, and varying differentiation assays make it difficult to compare studies and draw them into a consistent framework. Nevertheless, the similarities that exist suggest a common relationship between an archetypal CNS stem cell and its progeny (Figure 1). The data suggest that stem cells derived from different regions of the CNS display a similar growth factor responsiveness. Furthermore, regional differences in the brain appear to control the specific type of neurons that are formed.

Are there relationships among the various cells described above? All of the known multipotential neural stem cells, capable of forming both neurons and glia, express nestin. Nestin is subsequently expressed in glial derivatives of the NCSC and of CNS stem cells. Specifically, nestin is expressed in nonmyelinating Schwann cells, radial glia, and activated astrocytes. It is thus interesting to consider that these nestin-expressing glial derivatives may retain multipotential stem cell activities. It is also worth noting that stem cells may beget self-renewing cells of progressively limited developmental potential. In this light, cells such as the O-2A progenitor may truly be stem cell intermediates through which the cortical and striatal stem cells generate both astrocytes and oligodendrocytes. Similarly, a direct comparison of stem cells derived from embryos and those derived from adults will be important to examine the extent to which self-renewing stem cells from the latter retain the developmental potential of the former. Thus, relationships are bound to exist, but current studies only begin to address these issues.

How might one understand the origins of neural diversity in the developing vertebrate and its maintenance in the adult? To answer this question, it is important to realize that there are two conceptually separable issues: regional specification and stem cell differentiation. While progress is being made toward the molecular and cellular bases of both phenomena, the two have yet to be effectively integrated. It seems most fruitful to view the stem cell populations that constitute the primordial neuroepithelial sheet as the substrate on which patterning mechanisms act. Regional fate cannot be adopted in the absence of a multipotential stem cell. Likewise, for a stem cell to produce a particular neural or glial type, the appropriate positional signals (intrinsic and extrinsic) must be present. Hence, the problem becomes one of understanding how regional specification mechanisms interact with stem cell mechanisms to control differentiation.

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Neurons derived in vitro from ES cells express homeoproteins characteristic of motoneurons and interneurons

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Abstract

We have characterized different neuronal subpopulations derived from in vitro differentiation of embryonic stem (ES) cells using as markers the expression of several homeodomain transcription factors. Following treatment of embryoid-like aggregates with retinoic acid (RA), Pax-6, a protein expressed by ventral central nervous system (CNS) progenitors is induced. In contrast, Pax-7 expressed in vivo by dorsal CNS progenitors, and *erbB3*, a gene expressed by neural crest cells and its derivatives, are almost undetectable. CNS neuronal subpopulations generated expressed combinations of markers characteristic of somatic motoneurons (*Islet-1/2*, *Lim-3* and *HB9*), cranial motoneurons (*Islet-1/2* and *Phox2b*) and interneurons (*Lim-1/2* or *EN1*). Molecular characterization of neural phenotypes generated from ES cells should considerably facilitate the identification of new genes expressed by restricted neuronal cell lineages.

Keywords: Mouse embryonic stem cells; Neuronal differentiation; Retinoic acid; Motoneuron; Homeobox

1. Introduction

Recently, much interest has been focused on the generation of neurons from pluripotent cells as tools to study early steps of neuronal differentiation in vitro (Bain et al., 1995), to preselect gene trap clones for neuronal mutations (Baker et al., 1997), and to develop clinical strategies for transplanting neural precursor cells in the nervous system (Brüstle and McKay, 1996). In vitro neuronal differentiation will play an important role in these strategies. In this context, embryonic stem (ES) cells represent an interesting model.

Mouse embryonic stem (ES) cells are pluripotent cell lines derived from the inner cell mass of 3.5-day blastocysts of preimplantation mouse embryos (Evans and Kaufman, 1981; Martin, 1981). They are considered to be closely related to primitive ectoderm of the very early postimplantation embryo. ES cells can be maintained in an undifferentiated state in culture for many passages in the presence of leukemia inhibitory factor (LIF), an inhibitor of ES cell differentiation (Williams et al., 1988). ES cells have two main properties. First, they are accessible for genetic manipulation while retaining their ability to colonize the germline of mouse chimeras. Secondly, under appropriate conditions, ES cells are able to differentiate spontaneously in culture to generate various cell types. When ES cells are cultured in suspension in non-adhesive dishes, cell aggregation takes place giving rise to the formation of small spheres called embryoid bodies (EBs) in which cell differentiation can be followed by cellular expression of specific markers restricted to a given lineage.

Initially, EBs generate populations of cells that express genes characteristic of primitive endoderm and mesoderm. Subsequently, they can generate cells of the hematopoietic, endothelial, muscle, and neuronal lineages (Keller, 1995). The sequence of events leading to lineage commitment in vivo is often observed within EBs, suggesting that this in vitro model obeys to some extent the rules prevailing in the whole embryo (Rohwedel et al., 1994). However, different differentiation conditions can drastically affect the propor-

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tions of different cell types obtained in the EBs. In the absence of inducers, neurons represent only a small percentage of the total cells (Wobus et al., 1988). Unlike hematopoietic, endothelial and muscle cell lineages, efficient generation of neurons from EBs requires an additional inductive stimulus provided by retinoic acid (RA) which promotes neural and represses mesodermal gene expression in cultured mouse EBs (Bain et al., 1996) and in the P19 teratocarcinoma cell line (Jones-Villeneuve et al., 1982; Bain et al., 1994).

In these conditions, a neuroepithelial proliferating precursor cell population, which expresses the intermediate filament protein nestin, was derived from ES cells and can differentiate into neuronal (MAP2-positive) and glial (GFAP-positive) cells (Okabe et al., 1996). ES-derived neurons have been characterized by the expression of proteins associated with basic neuronal functions including III β-tubulin, neurofilament subunits, the neuronal surface marker N-CAM, transmitter synthesizing enzymes (GAD, TH, ChAT), transmitter receptor subunits (GluR), or by their electrophysiological properties (Bain et al., 1995; Strübing et al., 1995; Fraichard et al., 1995). While ES-derived neurons reveal electrophysiological and biochemical properties of CNS neurons (Strübing et al., 1995; Okabe et al., 1996), little is known of their precise phenotypes.

Motoneurons are among the first neurons formed. Their early development in vertebrates requires inductive action from the notochord and floor plate mediated by the secreted glycoprotein Sonic hedgehog (Shh) (for a review see Tanabe and Jessell, 1996). Shh inhibits Pax7 expression giving rise to ventralized CNS progenitors; secondly, graded Shh activity regulates Pax6 expression in progenitor cells, and thus influences the identity of neurons generated including Lim-1/2 interneurons and Islet-1/2 motoneurons (Ericson et al., 1996, 1997).

Over the last few years, a considerable number of markers have been used to distinguish neuronal subclasses within the CNS. In the developing spinal cord, the combinatorial expression of four transcription factors of the LIM family (Islet-1, Islet-2, Lim-1, and Lim-3) defines different motoneuron subtypes organized into longitudinal columns. Motoneurons located within a single column project their axons to a common peripheral target region (Tsuchida et al., 1994; Tosney et al., 1995). The two homeoproteins Islet-1 and Islet-2 are markers of motoneurons, although they are also expressed in other classes of neurons (Thor et al., 1991; Liem et al., 1997). In the brainstem, all cranial motoneurons seem to express Islet-1 whereas Islet-2 and Lim-3 are restricted to certain cranial motoneuron subpopulations (Varela-Echavarria et al., 1996). In the CNS, the Phox2b homeodomain transcription factor is expressed in cranial nuclei (except the VIth and XIIth) but not in spinal motoneurons. Cranial motoneurons in the hindbrain are classified into three distinct classes: somatic motor (SM) neurons innervate muscles derived from myotomes, branchiomotor (BM) neurons target the muscles developing from the branchial arches, and visceromotor (VM) neurons innervate autonomic ganglia. In the spinal cord, there are only SM and VM neurons. In the PNS, Phox2b, as well as the neuregulin receptor erbB3, is found to be expressed in the three cranial sensory ganglia (VIIth, IXth, Xth) and in all ganglia of the autonomic nervous system (Meyer and Birchmeier, 1995; Pattyn et al., 1997). The homeobox gene *HB9* (Harrison et al., 1994) is expressed in subsets of cranial (of the VIth and XIIth somatic motor nuclei; J. Livet, personal communication) and spinal motoneurons (Pfaff et al., 1996). The above markers can thus be used to characterize different motoneuronal subpopulations.

Here, we show that in the presence of RA, embryoid bodies derived from ES cells can differentiate into cell types characteristic of ventral CNS. These include at least two different subpopulations: an Islet-expressing population composed of somatic and Phox2b-expressing cranial motoneurons and an Islet-negative population probably composed of interneurons. Furthermore, we document the regulation of expression of several markers during in vitro maturation of neurons.

2. Results

2.1. Neuronal differentiation is regionalized within embryoid bodies derived from CCE embryonic stem cells

Some embryonic stem cell lines such as the D3 ES cell line require the use of feeder layers, which impose constraints on manipulation (Doetschman et al., 1985) whereas others, such as the CCE cell line, are feeder-independent (Robertson et al., 1986; Keller et al., 1993). Undifferentiated ES cells from the CCE ES cell line were grown on gelatin-coated (0.1%) tissue culture dishes in standard ES cell culture medium containing LIF (1000 units/ml). On day 0, ES cells were cultured in hanging drops distributed on non-adhesive bacteriological Petri dishes and incubated for 2 days at 37°C in 5% CO_2 (Strübing et al., 1995). ES cells continued to divide and aggregate at the bottom of drops, forming embryoid bodies (EBs) in which cell differentiation occurred. On day 2, hanging drops were resuspended in medium containing 2 μM RA and dishes were kept at 37°C for 5 more days. EBs treated with RA were collected at day 7 (EB7) and cell types were analyzed on cryostat sections by immunostaining. Neural precursor cells were stained using an antibody against the intermediate filament protein nestin (Fig. 1A) which is expressed in CNS and PNS precursors (Lendahl et al., 1990). Nestin-positive cells were distributed throughout the EBs. Since nestin is also expressed by muscle precursors (Sejersen and Lendahl, 1993), we used an antibody against the muscle-specific protein desmin to show that dissociated cells from RA-treated EBs did not include myoblasts (data not shown). Post-mitotic neurons were detected on sections at the periphery of EBs using antibodies directed either against the neurofila-

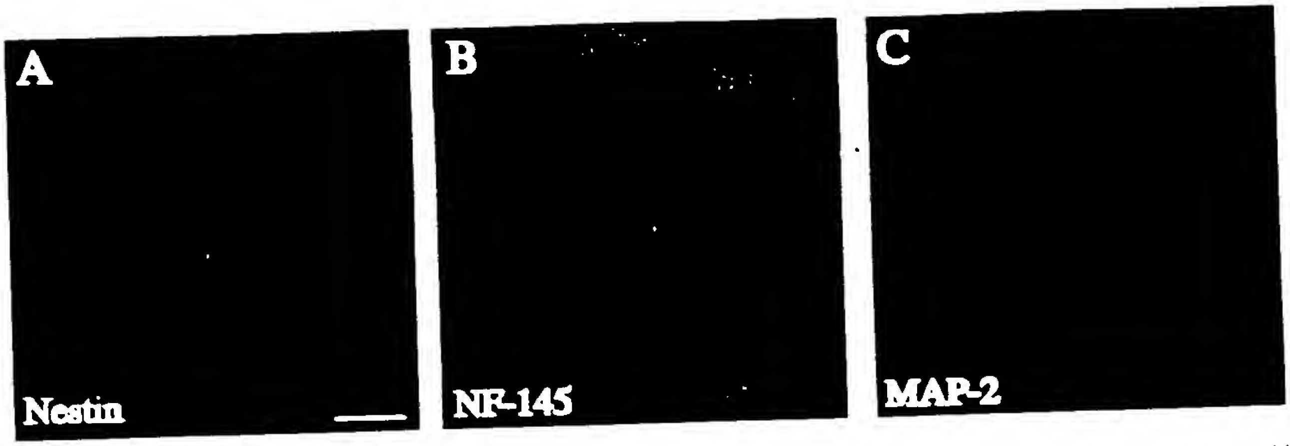

Fig. 1. Immunolabeling of neuronal cells in embryoid bodies (EBs). Immunostaining using three different antibodies on cryostat sections of RA-induced EBs fixed at day 7. In contrast to nestin-positive precursors (A), post-mitotic neurons detected by NF-145 (B) and MAP-2 (C) antibodies were preferentially observed at the periphery of EB. Scale bar, 200 μm.

ment NF-145 subunit (Fig. 1B) or the microtubule-associated protein MAP-2 (Fig. 1C). Thus, after seven days of culture, RA-induced EBs show the presence of neural progenitors as well as post-mitotic neurons.

2.2. *Analysis of neuronal gene expression in EBs by RT-PCR*

Gene expression was further analyzed in embryoid bodies by reverse transcription-polymerase chain reaction (RT-PCR) using a panel of different specific primers. RNAs were isolated at days 6 and 7 from non-induced and RA-induced EBs, then analyzed by semi-quantitative RT-PCR (Fig. 2). Members of the Trk family of tyrosine kinase receptors are expressed in most classes of neurons during their development (reviewed in Barbacid, 1994). We showed that the levels of *TrkC* and *TrkB* transcripts are up-regulated after RA treatment, as has already been shown for *TrkA* during neuronal differentiation of P19 embryonal cells (Salvatore et al., 1995).

We examined by RT-PCR the expression of three different neurotransmitter-synthesizing enzymes during EB differentiation. Transcripts of glutamic acid decarboxylase (*GAD*) are characteristic of GABAergic neurons, tyrosine hydroxylase (*TH*) of dopaminergic and noradrenergic neurons, and choline acetyltransferase (*ChAT*) of cholinergic neurons. Levels of their mRNAs were up-regulated in embryoid bodies by RA. Thus, our results show a striking induction by RA of neuronal markers in EBs during the 6th and 7th days of differentiation.

2.3. *RA-induced neurons from ES cells exhibit ventral CNS differentiation*

In vivo, the dorso-ventral polarity in the neural tube is reflected by domains of *Pax* gene expression. *Pax6* is expressed in ventral progenitors including motoneurons whereas *Pax7* is expressed in dorsal progenitors including neural crest cells (Mansouri et al., 1996; Ericson et al., 1997). In order to better characterize the different types of neuronal sub-populations induced in vitro, we examined by immunostaining the expression of *Pax6* and *Pax7* genes on cryostat sections of embryoid bodies at day 7 (EB7). Whereas *Pax6* was not expressed in undifferentiated ES

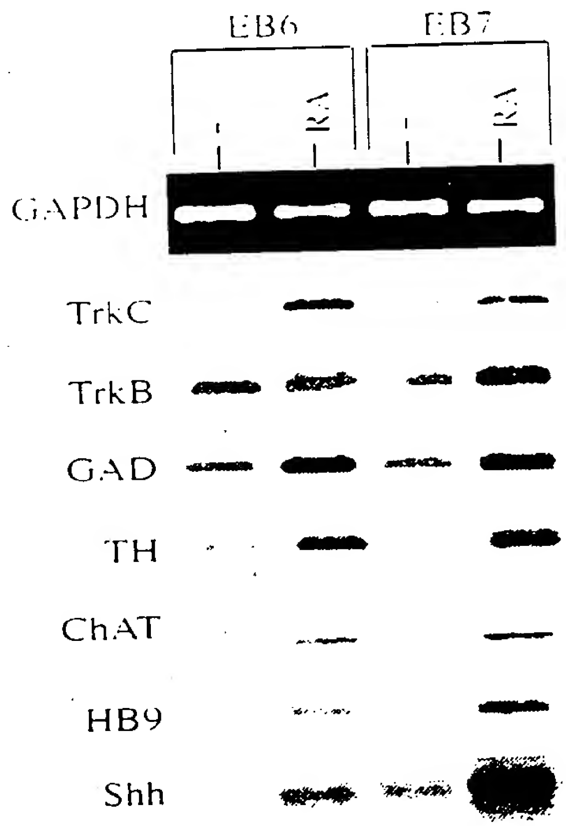

Fig. 2. Up-regulation of neuronal gene expression following RA treatment. RT-PCR assays were performed on embryoid bodies harvested at day 6 (EB6) or day 7 (EB7). RNAs were isolated from non-induced and from RA-induced EB6 and EB7. RA up-regulates the levels of transcripts for *TrkC* and *TrkB* receptors, the three neurotransmitter-synthetizing enzymes (*GAD*, *TH* and *ChAT*), the *HB9* transcription factor and the signaling molecule *Shh*. RT-PCR assays were performed and PCR products were hybridized to DIG-dUTP specific probes (see Section 4).

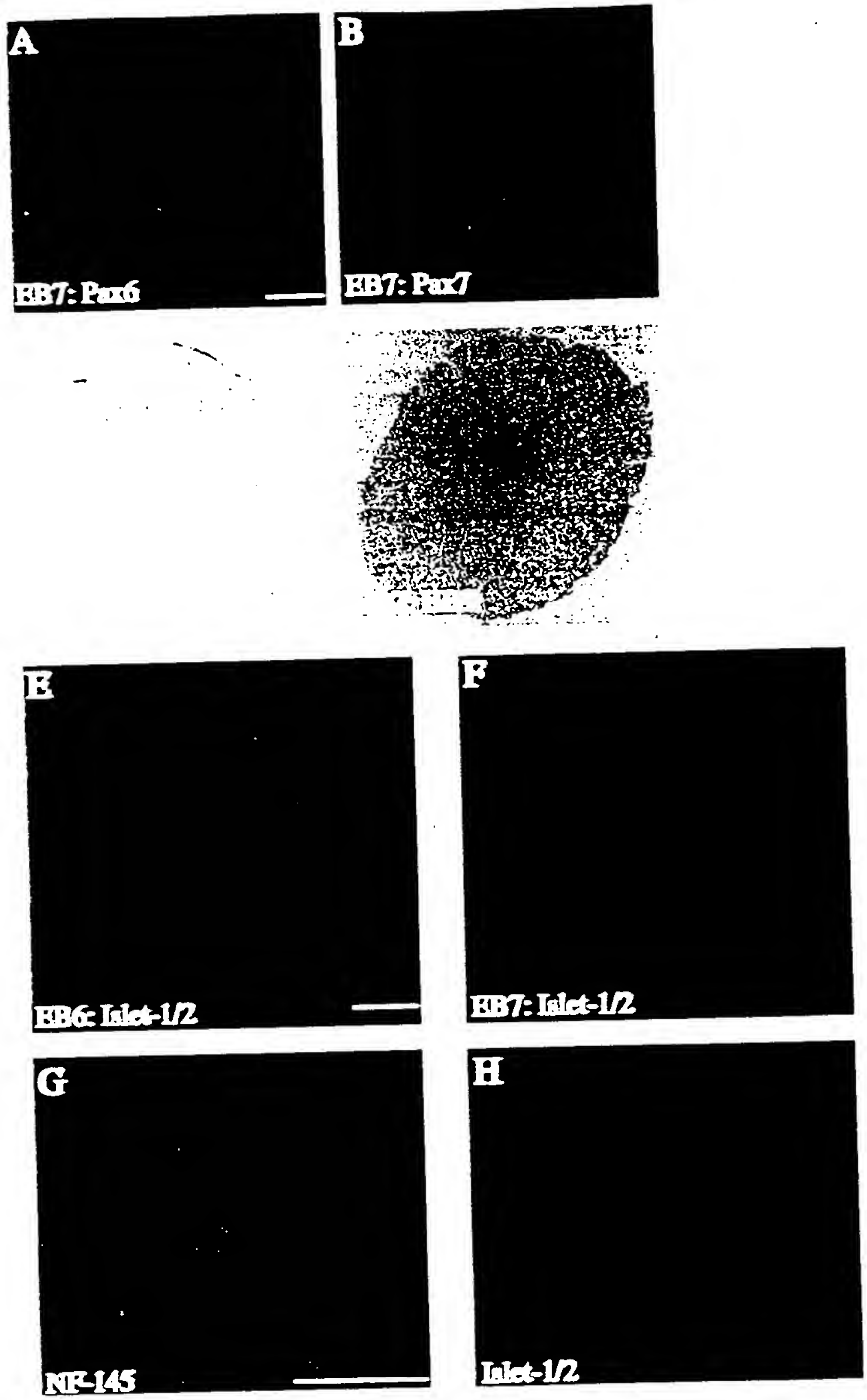

Fig. [illegible]. Expression of CNS markers in embryoid bodies (EBs) and plated neurons. Cryostat sections of RA-induced EB6 and EB7 were immunostained for Pax6 (A), Pax7 (B), or Islet-1/2 (E,F). Pax6-positive cells are located in the central region of EB (A) but only a few cells express Pax7 at low levels (B). No signal was obtained by ISH on EB7 using an *erbB3* riboprobe (C) whereas a strong signal was observed following ISH in sections of E14.5 dorsal root ganglia (not shown). HB9-expressing cells are detected in EB7 (D). The number of Islet-positive cells in EB increases between day 6 (E) and day 7 (F). In other experiments, EB7 were dissociated, cells were plated and double-immunostained 24 h later for NF-145 (G) and Islet-1/2 (H). Scale bars, 200 μm.

cells (data not shown), we found clusters of Pax6-positive cells preferentially located within the central region of EBs (Fig. 3A). In contrast, *Pax7* expression was almost undetectable (Fig. 3B). These results suggest that in these conditions RA induces central nervous system progenitors of ventral character, almost to the exclusion of dorsal neural tube progenitors. In order to confirm these data, we used in situ hybridization (ISH) to detect the expression of the *erbB3* neuregulin receptor transcripts, which are expressed predominantly in the peripheral nervous system (Meyer et al., 1997; our unpublished data). Expression of *erbB3* is found in peripheral ganglia cells derived from the neural crest as well as Schwann cell precursors but not in the central nervous system (Meyer and Birchmeier, 1995). No erbB3 mRNA was detected in EB7 (Fig. 3C). In vivo, the graded signaling activity of Sonic hedgehog (Shh) controls the generation of distinct classes of motoneurons and ventral interneurons in the neural tube (Ericson et al., 1997). Using RT-PCR, we demonstrated that *Shh* transcript levels in EBs were up-regulated by the addition of RA at the time when Pax6-positive cells are detected within EBs (Fig. 2). We propose that this could be one reason why in vitro neuronal differentiation of EBs generates ventral CNS progenitors.

Since motoneurons express the homeoproteins Islet-1 and/or Islet-2, we asked whether motoneurons were being generated in RA-treated EBs by following Islet expression on EB cryostat sections at days 6 and 7. We used an antibody that recognizes both Islet-1 and Islet-2 proteins; the staining obtained with this antibody will be referred to as Islet staining. On day 6, on sections of EBs induced by RA, we detected a few Islet-positive cells preferentially localized at the periphery (Fig. 3E), whereas by day 7 this number had dramatically increased (Fig. 3F). To see if Islet-positive cells were neurons, EB7 were collected and disaggregated cells were seeded in complete F12 medium without RA. Within a few hours, the first neurite processes began to extend. The cells were fixed for immunohistochemistry 24 h later. Double immunostaining on dissociated cells using NF-145 (Fig. 3G) and Islet antibodies (Fig. 3H) showed the presence of Islet-positive neurons, as well as non-neuronal Islet-expressing cells (not shown).

To assess the efficiency of induction of neurons and in particular Islet-positive neurons in our system, we performed double immunostaining on dissociated cells using NF145 and Islet antibodies and counted the numbers of NF-positive/total cells and Islet-positive/NF-positive cells. A variety of batches of RA from different suppliers was tested for the efficiency of Islet induction. The batch used for all experiments described in this paper induced a cell population in which 10% were neurons (i.e. NF-positive) and between 40 and 68% of those neurons were Islet-positive. Certain batches of RA were found to give reproducibly higher proportions of Islet-positive neurons, although this was accompanied by a reduction in the total number of neurons induced. Since the availability of starting material, i.e. ES cells, is non-limiting, this proportion of neurons did not hinder the generation of different neuronal subpopulations in sufficient numbers for quantification.

2.4. ES cell-derived neuronal sub-populations induced in vitro

We further characterized the neuronal populations produced in our differentiation system, using combinations of markers of cranial and spinal cord neuronal subpopulations.

2.4.1. Somatic motoneurons

In order to characterize unambiguously the presence of somatic motoneurons after in vitro neuronal differentiation, we tested the expression of HB9, which in the neural tube is restricted to spinal and cranial somatic motoneurons. We showed by RT-PCR that *HB9* transcripts are not detected at significant levels in the absence of RA, whereas addition of RA up-regulates their expression in EB6 and EB7 (Fig. 2). HB9-expressing cells were detectable by in situ hybridization on EB7 cryostat sections (Fig. 3D). Correspondingly, we observed the presence of large HB9-positive neurons in dissociated cultures from EB7. By double immunostaining using Islet (Fig. 4A) and HB9 (Fig. 4B) antibodies on dissociated cells, we showed that all HB9-positive neurons expressed Islet, and that they represented 12% of the Islet-positive neurons (Fig. 5). In contrast, no cells were detected that co-expressed HB9 and Phox2b, a marker for non-somatic cranial motoneurons (Fig. 5). We suggest that the HB9-positive cell population is composed of somatic motoneurons that may be cranial and/or spinal.

Induction of the somatic motoneuron phenotype by RA was further confirmed by double immunostaining using Islet (Fig. 4D) and Lim-3 (Fig. 4E) antibodies. In the chick, *Islet-1* and *Lim-3* markers co-localize in subsets of cranial somatic motoneurons and also in the median part of the median motor columns (MMCm) from spinal cord (Tsuchida et al., 1994; Varela-Echavarria et al., 1996). Low numbers of doubly stained neurons were detected in our cultures. Another subset (3% of the Islet-1-positive neurons; see Fig. 5) of neurons was detected by double immunostaining using Islet-1 (Fig. 4G) and Lim-1/2 (Fig. 4H) antibodies. In chick explants, a few such double-positive cells were also detected transiently at the junction of the motoneuron and interneuron (Lim-1/2-positive) populations (Ericson et al., 1996). We observed that all Islet-1-positive neurons were also detected by the anti-Islet-1/2 antibody (Fig. 5). This suggests that somatic motoneurons generated from ES cells do not include motoneurons (Islet-2-positive and Islet-1-negative) which belong to the LMCl subpopulation (Tsuchida et al., 1994).

2.4.2. Phox2b-expressing cranial motoneurons

We asked the question whether RA-induced motoneurons were preferentially of cranial or spinal subtypes. To answer this question, we used an antibody against Phox2b, a homeodomain transcription factor expressed in cranial somatic

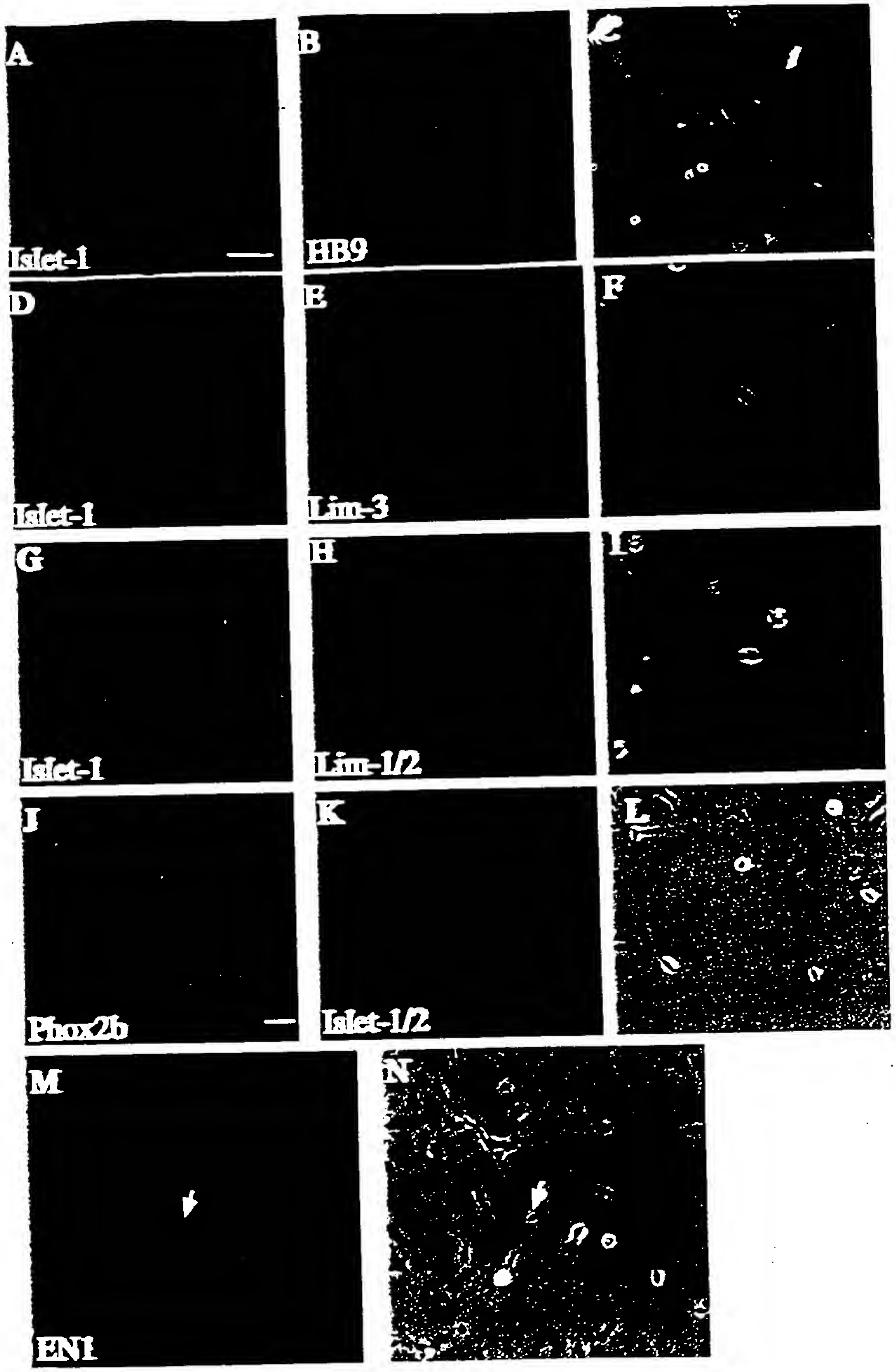

Fig. [illegible] cell-derived CNS neuronal subpopulations express markers characteristic of motoneurons. (A–L) RA-induced [illegible] were dissociated, cells were plated and double immunostained 24 h later for the indicated antigens. Panels C, F, I and L are phase contrast photomicrographs. (M,N) Single labeling for EN1 and corresponding phase contrast field. Scale bar, [illegible] μm.

motoneurons (except for the VIth and XIIth nuclei), non-somatic cranial motoneurons such as branchiomotor (BM) and visceromotor (VM) neurons but not in spinal motoneurons. By double immunostaining on dissociated cells using anti-Phox2b (Fig. 4J) and anti-Islet (Fig. 4K) antibodies, we found that about 60% of Islet-positive neurons expressed Phox2b (Fig. 5). We conclude that most of the motoneurons generated from ES cells in our system are of cranial subtype.

2.4.3. *Interneuron subtypes*

In vivo, Lim-1 and Lim-2 are expressed in interneurons throughout the rostrocaudal extent of the developing brain stem and spinal cord (Varela-Echavarria et al., 1996; Tanabe and Jessell, 1996). However, apart from Lim-1 in the lateral motor columns of the limb (LMCl) (Tsuchida et al., 1994), they are not expressed in motoneurons. By double immunostaining on dissociated cells using Islet and Lim-1/2 antibodies, we found that more than 95% of Lim-1/2-positive neurons were Islet-negative (Fig. 5), suggesting that they were interneurons. We also detected low numbers of neurons that expressed the homeodomain protein Engrailed1 (EN1) (Fig. 4M). In vivo, EN1-positive interneurons are located close to motoneurons, but *EN1* and *Islet* markers are not coexpressed by single neurons in the spinal cord (Pfaff et al., 1996).

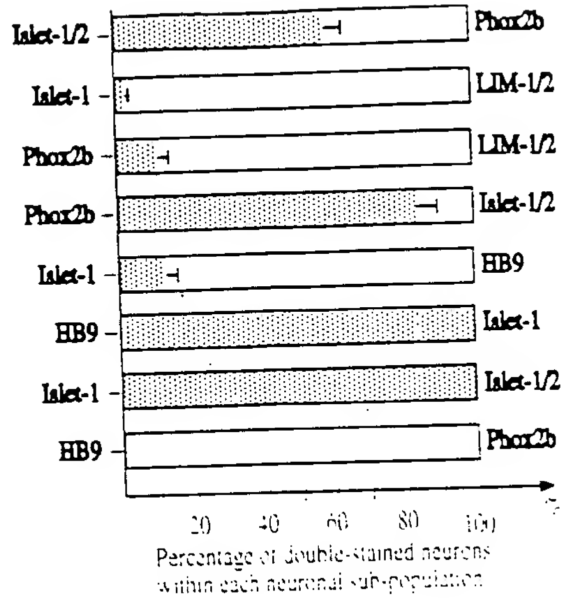

Fig. 5. Relative proportions of neuronal subpopulations induced in [illegible]. Doubly-labeled cells as in Fig. 4 were quantified by direct counting of [illegible] 300–400 neurons per condition in 2–4 independent experiments. The proportion of neurons positive for one antigen (left, [illegible] type) that also expressed a second marker [illegible] is shown by the [illegible] bar; mean ± SEM.

2.5. *Regulation of expression of several neuronal markers during in vitro differentiation*

We next determined the timing and sequence of appearance of several markers characteristic of different stages of motoneuron differentiation: *nestin, Islet, peripherin, ChAT* and *VAChT*.

We carried out double immunostaining for NF-145 and nestin on 24 h cultures of dissociated EBs. Post-mitotic NF-145-positive neurons (Fig. 6A) and nestin-positive precursor cells (Fig. 6B) were observed. Expression of both markers is tightly regulated in RA-induced cell cultures since in NF-positive differentiated cells, nestin protein is either down-regulated or completely absent. In most nestin-positive precursor cells (see the pair of nestin-positive cells in Fig. 6B), the NF-145 subunit is not detectable (see Fig. 6A). These results show that the transition from nestin expression in neuronal precursors towards neurofilament expression in differentiated neurons observed during normal development can be reproduced in vitro during neuronal differentiation of ES cells.

Peripherin is a type III intermediate filament that has been shown to be expressed in motor, sensory and sympathetic neurons (Djabali et al., 1991). By double immunostaining with peripherin (Fig. 6C) and Islet (Fig. 6D) antibodies of RA-induced day 7-dissociated cells after 24, 48 and 72 h in culture, we determined the proportion of peripherin-positive neurons within the Islet-positive cell population. Whereas the percentage of Islet-positive cells relative to the total number of cells was maximum at 24 h (data not shown), the expression of peripherin was progressively up-regulated between 24 h and 72 h. About 15% of the Islet-positive cells were peripherin-positive after 24 h whereas this proportion reached almost 70% after 72 h in culture (Fig. 7). This is consistent with what is observed in vivo, where NF-L expression occurs before that of peripherin (Escurat et al., 1990).

Motoneurons are cholinergic and express the biosynthetic enzyme choline acetyltransferase (ChAT) and the vesicular transporter for acetylcholine VAChT. Both markers co-localize in cholinergic neurons and are expressed late during development when synaptic contacts are established (Arvidsson et al., 1997). By single immunostaining with ChAT (not shown) or VAChT (Fig. 6E) antibodies, we showed that both markers are expressed in neurons induced by RA, but we never detected their presence by immunostaining before seven days of culture after EB dissociation (data not shown). Both markers are thus tightly regulated during in vitro neuronal differentiation.

3. Discussion

We show here that treatment with retinoic acid in appropriate conditions can cause ES cells to differentiate into different neuronal subclasses characteristic of the ventral CNS including somatic motor, branchiomotor or visceromotor neurons and ventral interneurons. Precise molecular characterization of neural cell phenotypes induced by differentiation of ES cells should considerably facilitate iden-

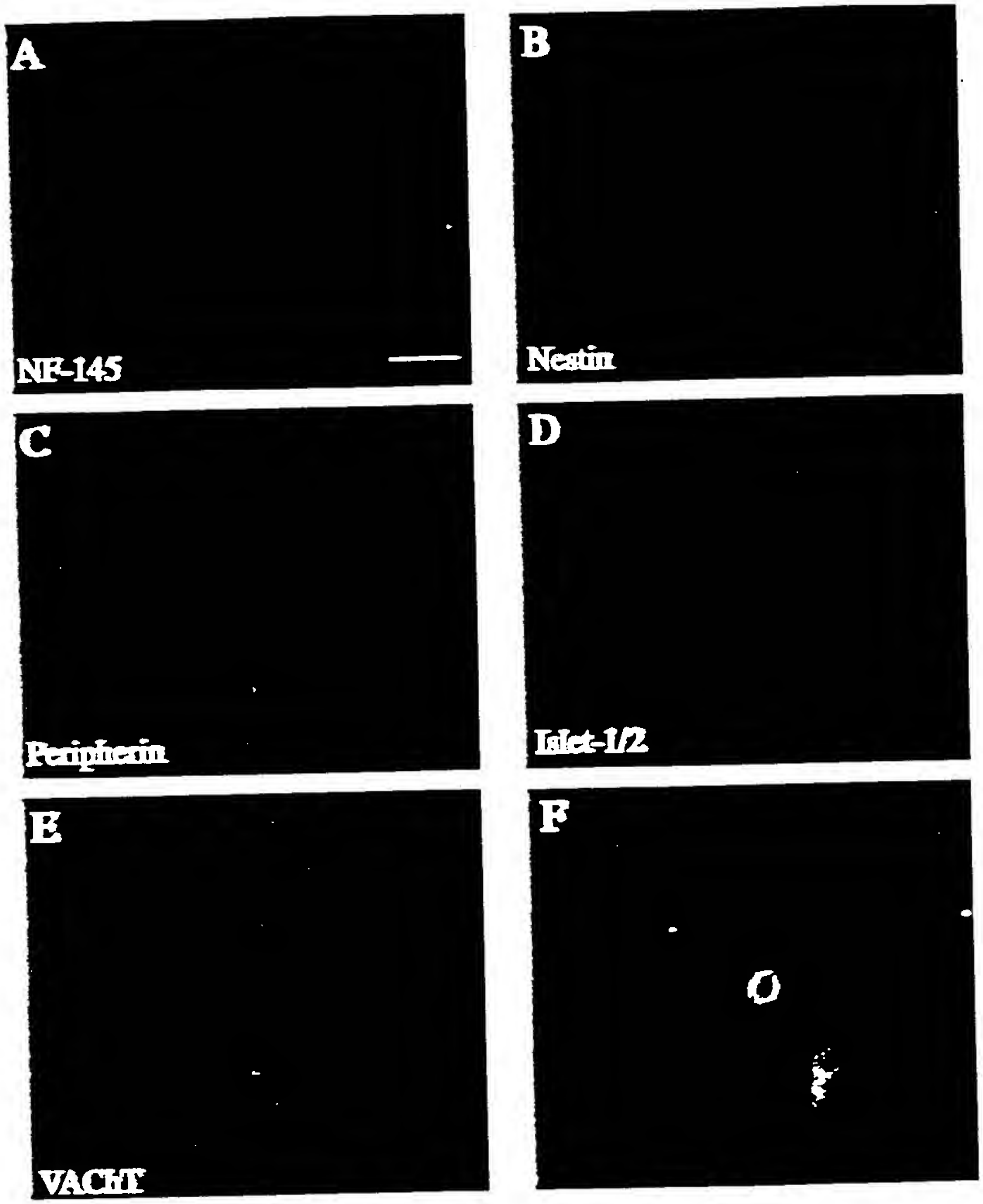

Fig. [illegible] In vitro maturation of ES cell-derived neurons. Double [illegible] for the indicated antigens. Note the coexpression of nestin and NF-145 within a single cell surrounded by [illegible] EB7 were dissociated and immunolabeled 7 days later using antibodies against VAChT. Scale [illegible]

tification of genes differentially expressed in these neuronal subpopulations.

Using several markers of CNS or PNS neural progenitors, together with markers of defined neuronal subpopulations, we have tried to define the identity of neurons produced from ES cells. Several lines of evidence presented here indicate that RA induces the CCE ES cell line to differentiate into CNS neurons of ventral character. In mammals, *Pax6* expression is restricted to the CNS, and in the neural tube its expression domain is in the ventral region (Walther and Gruss, [illegible]; [illegible] et al., [illegible]). The expression of *Pax6* in ES cell-derived embryoid bodies (Gajovic et al., [illegible]) [illegible] that of *erbB3* suggests that ES cell-derived [illegible] the CNS type and not PNS type. Furthermore, [illegible] expression and the almost complete absence of the [illegible] marker *Pax7* in EBs argues for the generation of CNS neurons of ventral character. However, we cannot completely exclude the possibility that dorsal precursors are generated since some cells expressing very low levels of Pax7 were observed. Finally, up-

regulation of the expression of Shh by RA in our differentiation system fits with the notion of ventral CNS differentiation. Shh is expressed by the floor plate along the rostro-caudal axis of the vertebrate embryo and plays an essential role in the differentiation of ventral neuronal subtypes (reviewed in Tanabe and Jessell, 1996). Interestingly, RA, acting through a RARE in the Shh promoter has been shown to directly activate transcription of the *Shh* gene in HeLa cells (Chang et al., 1997). Other reports have already suggested that, after RA treatment, ES cell-derived neurons show electrophysiological and biochemical properties of postmitotic CNS neurons (Strübing et al., 1995; Okabe et al., 1996). Neurons derived from the BLC 6 ES cell line express receptors for the main inhibitory (GABA and glycine) and excitatory (glutamate) neurotransmitters in the CNS (Strübing et al., 1995). Similar sets of currents have been detected in neurons derived from the ES cell lines CCE and D3 when differentiated after RA treatment (Bain et al., 1995). It is striking that in all these different procedures, neuronal differentiation always requires between 5 and 6 days after the addition of RA in order to produce the first post-mitotic neurons.

We characterized different neuronal subtypes within the cell population induced by RA, by investigating the expression of genes representing several families of homeodomain proteins, the expression profiles of which can be used to define cranial and spinal cord neuronal subpopulations. After EB dissociation at day 7 and cell plating, the ES cell-derived neuronal cell population can be divided in two classes: one expressing Islet and a second class expressing Lim-1/2 and not Islet. The Islet-expressing cell population is composed of two classes of somatic motoneuron. The HB9-positive cranial and/or spinal motoneurons and Lim-3-positive motoneurons which could be either cranial or spinal from the median motor columns (MMCm), and presumptive cranial SM, BM or VM neurons which are Phox2b-positive. It is noteworthy that in our system at least 60% of RA-induced motoneurons generated from ES cells are of the cranial subtype. Several effects of RA on CNS antero-posterior patterning have been reported in vivo within the hindbrain (Lumsden and Krumlauf, 1996; Blumberg et al., 1997). During gastrulation, the development of the vertebrate hindbrain is sensitive to excess of RA, which causes apparent transformation of anterior structures toward more posterior identities (Maden and Holder, 1992). Vitamin A-deficient quail embryos present severe defects in their CNS including absence of posterior hindbrain (Maden et al., 1996). Recent evidence shows also that retinoid receptor signaling promotes primary neurogenesis in Xenopus (Sharpe and Goldstone, 1997). However, both in vitro and in vivo, it remains unclear how RA induces neurogenesis. Although several effects of RA described in vivo in the hindbrain may be correlated to our in vitro data showing the induction of cranial motoneurons, the molecular mechanisms underlying such aspects in vitro remain to be defined. Evidence for more specific roles for retinoids via RAR and/or RXR receptors in motoneuron development was provided in a recent report by Sockanathan and Jessell (1998). These authors demonstrated that RA causes specific proliferation of spinal cord precursors that further differentiate towards a motoneuronal pathway and also clearly established that RA later participates in the specification of LMCl motoneurons. The absence of the LMCl motoneuron subtype in our system might reflect the possibility that the somatic motoneurons we observe are of the cranial subtype and that the calculated percentage of cranial motoneurons (60%), based on Phox2b expression, may be an underestimate. It would be interesting, in light of our results to examine whether particular retinoid receptors also participate in the specification of cranial motoneurons in vivo.

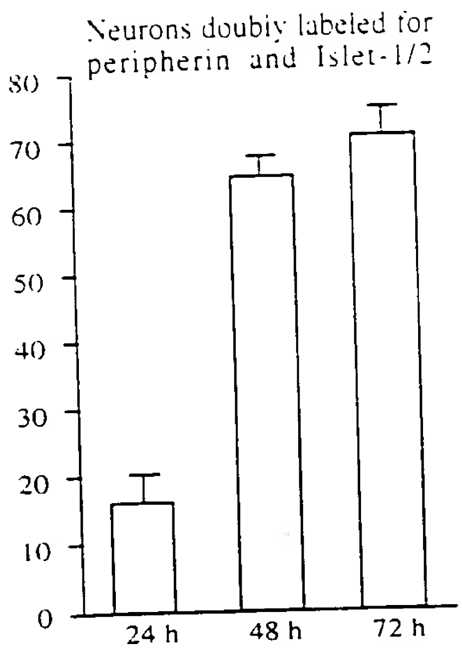

Fig. 7. Time-course of peripherin expression by ES cell-derived Islet-1/2 neurons. We calculated the proportion of peripherin-positive neurons within the Islet-positive cell population in dissociated cultures at 24, 48 and 72 h. During differentiation, the number of neurons expressing both markers increases significantly.

The expression pattern of Lim-1/2 interneurons during neurogenesis defined by Varela-Echavarria et al. (1996) demonstrated Lim-1/2 neurons distributed throughout the dorso-ventral neural tube. Thus, the Lim-1/2-expressing neurons observed in our system that were Islet-negative were taken to be interneurons. As in vivo (Pfaff et al., 1996; Ericson et al., 1997), a small number of neurons expressed EN1. The class of Islet-positive dorsal interneurons, termed D2 neurons (Liem et al., 1997) was apparently not induced, like other dorsal CNS populations.

Our study thus represents the first demonstration of an in vitro differentiation system for ES cells which reproducibly generates particular classes of neurons, in this case Islet-expressing neurons including motoneurons and Lim-1/2-expressing neurons including interneurons. In vivo, these neuronal subpopulations have been shown to be produced from ventral Pax7-negative progenitors by Ericson et al.

(1996). It is therefore tempting to speculate that Pax6 and Pax7 gene regulation is also predictive of the generation of Islet-positive and Lim-1/2-positive phenotypes in vitro.

We showed that the expression of neuronal markers is tightly regulated in RA-induced cell cultures in which the transition from nestin expression in neuronal precursors towards neurofilament expression in differentiated neurons can be reproduced. Whereas the percentage of Islet-positive cells relative to the total number of cells was maximal at 24 h in vitro after EB7 dissociation, the expression of peripherin, a marker of more mature neurons including cranial motoneurons, was progressively up-regulated during the first 3 days after EB dissociation (Escurat et al., 1990; Pattyn et al., 1997). We observed a cholinergic phenotype for the neurons remaining after 7 days of culture by single immunostaining with ChAT (not shown) or VAChT (Fig. 6E) antibodies. We could not directly associate this mature cholinergic phenotype with the 24–72 h old Islet-expressing neurons because we could not detect VAChT or ChAT by immunolabeling before 7 days of culture. It could be that certain signaling interactions are missing in vitro since we also failed to detect ChAT or VAChT expression by purified E14 rat motoneurons cultured for 72 h (not shown). We cannot exclude that the failure to detect these markers by immunohistochemistry in younger neurons was due to technical problems due to very low levels of expression, since ChAT mRNA was readily detectable by RT-PCR in EB7.

The present data show that multiple steps of the in vivo development of CNS neurons can be reproduced in culture. This system will be a useful tool to study the effects of molecules (Shh, vitronectin, NT-3, BDNF) known to be involved in the generation of ventral neural tube cell populations (manuscript in preparation). Another promising approach is the creation of a bank of gene-trap ES clones prescreened by in vitro differentiation of ES cells (Baker et al., 1997). The diversity of characterized neuronal phenotypes obtained in our system, suggest that in vitro preselection will be helpful in identifying new genes expressed in restricted areas of the CNS.

4. Experimental procedures

4.1. Cell culture and media

The ES cell line CCE (Robertson et al., 1986) was maintained in ES medium: DMEM, 15% FBS (fetal bovine serum from GibcoBRL no. 10270), 2 mM L-glutamine, 1 mM Na pyruvate, 10 units/ml penicillin, 10 µg/ml streptomycin, 0.1 mM β-mercaptoethanol, leukemia inhibitory factor (Gibco-Esgro LIF) at 1000 units/ml. During the first 2 days, ES cells were cultured in 50 µl hanging drops containing 2500 ES cells (Rohwedel et al., 1994). Embryoid bodies (EBs) medium contains the same components as ES medium except for 20% FBS, no LIF and no β-mercaptoethanol. A 10-cm Petri dish was placed upside-down on its lid, together with a 3.5-cm dish containing 2 ml of PBS to prevent evaporation from hanging drops. All-trans-retinoic acid (Sigma, R 2625) was added at 2 µM at day 2. The medium containing RA was prepared in the dark. The medium was not changed between days 2 and 7. On day 7, EBs were sedimented within a few minutes in a tube containing 10 ml PBS at 37°C. EBs were washed once with PBS and dissociated. ES-derived neurons were grown on 35-mm polyornithine/laminin-coated dishes in 2 ml of F12 complete medium containing BSA (0.3% w/v), FBS (2%), L-glutamine (2 mM), penicillin (10 units/ml), streptomycin (10 µg/ml), transferrin (0.1 mg/ml), putrescine (16 µg/ml), Na selenite (160 ng/ml), insulin (10 µg/ml), progesterone (60 ng/ml).

Culture dishes were precoated during 2 h with 2 ml of 3 µg/ml of polyornithine (PORN) diluted in water and after removing PORN, they were dried for 30 min. Two milliliters of basal F12 medium containing 3 µg/ml of laminin (LN, Becton Dickinson) were added and the dishes were incubated overnight at 37°C, 5% CO_2. The medium was changed quickly to culture medium containing dissociated cells, whilst not allowing the laminin to dry.

4.2. EB dissociation

To facilitate quantitative analysis of the neuronal types present, EBs were dissociated following a protocol found to minimize cell death. EBs were gently pipeted up one by one into a 1 ml blue tip in a minimal quantity of medium and collected in a transparent tube containing 10 ml PBS. EBs were never centrifuged but were allowed to sediment at the bottom of the tube for a few minutes. Between 40 and 50 EBs were recovered per dish. EBs were washed in PBS at 37°C and dissociated by adding 500 µl of 0.25% [illegible] trypsin-EDTA which was vortexed very gently for 5 min. EBs were dissociated by slowly pipetting the solution 10–15 times into a 1-ml blue tip. After EB sedimentation, 400 µl of the supernatant containing dissociated cells were recovered and transferred to medium containing 20% FBS. After adding a second 400 µl volume of 0.25% trypsin-EDTA to the EB pellet, the dissociation was repeated; most dissociation occurred during the third and fourth rounds, although dissociated cells were obtained earlier. The complete dissociation should not exceed 35–40 min because massive cell death occured. Cells were centrifuged at 270 g for 5 min and resuspended in 4 ml of F12 complete medium. Efficient dissociation of 40–50 EBs gave normally between 7×10^5 and 9×10^5 healthy cells.

4.3. Embryoid body cryostat sections

EBs were collected at day 6 (EB6) and 7 (EB7) of culture, washed in PBS, fixed in 4% paraformaldehyde for 2 h at 20°C and incubated in PBS 20% sucrose overnight. Cryostat sections (20 µm) of EB pellets were dried onto microscope slides for 30 min and slides were kept frozen before immu-

nostaining. Sections were permeabilized with Triton 0.1%, lysine 50 mM in PBS at 4°C for 15 min and then washed three times with PBS.

4.4. Antibodies and immunocytochemistry

Cells plated on 3.5-cm polyornithine/laminin coated dishes were washed in basal F12 and fixed with 4% paraformaldehyde, 0.1% glutaraldehyde in PBS at 4°C for 15 min. Fixed cells were washed in cold PBS before permeabilization in 0.1% Triton, 50 mM lysine in PBS at 4°C for 15 min. After washing in PBS, cells were incubated with blocking solution containing 2% BSA, 2% heat-inactivated horse serum, 50 mM lysine in PBS at 4°C overnight. Cells were then washed with PBS and incubated with primary antibodies used in double immunostaining for 2 h at RT (except for anti-HB9, 24 h at 4°C and anti-VAChT, 4 h at RT). Secondary antibodies donkey anti-rabbit FITC-conjugated (1:100), biotin-SP-conjugated donkey anti-goat IgG (1:200, Jackson ImmunoResearch Lab.) and Cy3-conjugated donkey anti-mouse at 1:400 (Jackson ImmunoResearch Lab.) were diluted in PBS, 2% horse serum, 8% FBS, 4% inactivated sheep serum, 2% BSA and applied to cells for 2 h at 20°C in the dark. For VAChT staining, we used Cy3-conjugated streptavidin (1:1000) incubated for 30 min in the same buffer after removing the secondary antibody. After three washes in PBS, cells were mounted in Citifluor. EB sections were immunostained as described for cultured cells except primary antibodies were incubated 3 h and longer washes in PBS were required after each incubation with antibodies.

Primary antibodies and dilutions in the same buffer used for secondary antibodies were: rabbit anti-NF-145 (1:500, Chemicon), rabbit anti-peripherin (1:200, a gift from M.M. Portier), rabbit anti-Islet-1 (1:200, A8, a gift from T. Jessell), rabbit anti-Phox2b (1:1000, a gift from C. Goridis), goat anti-VAChT (1:1000, Chemicon), mouse monoclonal anti-MAP2 (1:250, Sigma), monoclonal anti-HB9 (1:2, a gift from T. Jessell) monoclonal anti-Lim-1/2 (1:2, 4F2, a gift from T. Jessell), monoclonal anti-Lim-3 (1:100, 4E12, a gift from T. Jessell), monoclonal antibodies from Developmental Studies Hybridoma Bank: anti-nestin (1:100, rat-401), anti-PAX6 (1:100), anti-PAX7 (1:100), anti-EN1 (1:100, 4G11). 'Anti-Islet-1/2' was a mixture of two antibodies (from Dev. Studies Hybridoma Bank): 4D5 (1:200 on cultured cells; 1:100 on sections) and 2D6 (1:2). For double immunostaining, negative controls were performed by omitting one of the two primary antibodies or one of the two secondary antibodies. Positive controls were performed using either purified E14 rat motoneurons, E14 rat dissociated spinal cord or rat embryo sections.

4.5. RT-PCR assays

Total RNA was extracted from 100 to 150 EBs by the method of Chomczynski and Sacchi (1987) for each induction experiment. One microgram of total RNA was mixed with 250 ng oligo dT-anchor and the mixture was heated for 10 min at 70°C, chilled on ice and adjusted to 1× first-strand buffer. The cDNA synthesis was performed according to the instructions for Superscript II RNase H⁻ reverse transcriptase (GibcoBRL) in a final volume of 20 μl containing 10 mM DTT, 0.5 mM dNTPs, 10 units RNasin, and 200 U Superscript. Tubes were incubated for 1 h at 45°C, 5 min at 95°C, spun, and chilled on ice. PCR reactions were performed in a final volume of μl. Two microlitres of cDNA solution were amplified in 1× Taq buffer with 50 pmol of each primer, 100 μM dNTPs, and 2.5 U Taq polymerase (GibcoBRL). Thirty cycles of amplification were carried out and, unless otherwise indicated, cycle times were 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by 60°C for 2 min and 72°C for 5 min. Mouse primer sequences and the length of amplified products were as follows (forward primers are noted 's' for sense and reverse primers are noted 'as' for antisense):

TrkC, 56°C s1: TTCTGGAATTCGGAACATC,
as1: GGACCGGGATCCATACTTG, 1044 bp
as2: CATTGTACAGCCAGTGGAGC, 679 bp
TrkB, 57°C s1: CGTGGAGGAAGGAAAGTCTG,
as1: CGTGGAGGGGATTTCATTACT, 637 bp
s2: TCCAACCTCAGATCACCACTG,
as2: TCTGTCTCTCATCCTTCCCATA, 230 bp
HB9, 58°C s1: GCACAGGCCCCTCTCTATGG
as1: GTCTTGGAGGAGGCGAGTCG, 721 bp
s2: CCCTGCCGACCCCATCAA
as2: GCCTTGTCCCCCGAAACC, 441 bp
GAPDH, s1: ACGACCCCTTCATTGACCTCAACT
as1: ATATTTCTCGTGGTTCACACCCAT, 320 bp
TH s1: TGTCAGAGGAGCCCGAGGTC
as1: CCAAGAGCAGCCCATCAAAG, 412 bp
GAD: 63°C s1: GGACAGCAGGACAGCAAGA
as1: CTGGCAGACCCCGTGATAG, 426 bp
ChAT s1: TGGCAACTTCGTCGGAGGCTC
as1: CCTACGGATAGGACCTTTTCCA, 217 bp
Shh s1: GCACCCCAAAAAGCTGACCC
as1: CGTGGTGATGTCCACTGCTCG, 349 bp
s2: AGAGGCGGCACCCCAAAAAG
as2: TCATCCCAGCCCTCGGTCAC, 296 bp

PCR products generated from EBs were separated on 1% agarose gels and transferred onto Hybond N+ membrane in 0.4 M NaOH. The filter was washed for 10 min in 2× SSPE and irradiated by UV. Filters were incubated in prehybridization buffer (6× SSC, 5× Denhardt, 500 μg/ml denaturated salmon sperm) at 65° for 1 h and then hybridized overnight with DIG-dUTP probes in the same buffer. Specific probes were generated from PCR products by using mouse neural tube cDNA as template. Probes were purified from 1% agarose gels on Qiaquick columns (Qiagen). The specificity of the amplified sequences were verified either by restriction mapping or Southern blotting using nested PCR products as probes. A second round of amplification by PCR incorpor-

ating DIG-dUTP was then performed and probes were purified on columns. After hybridization, filters were washed twice in 2x SSC at 20°C for 5 min and twice in 0.2x SSC at 60°C for 15 min. After incubation in buffer 1 (150 mM maleic acid, 100 mM NaCl, pH 7.5) containing 1% blocking reagent (Boehringer), anti-DIG phosphatase alkaline linked antibody (1:4000, Boehringer) was applied on filters for 1 h at 20°C. After two washes in buffer 1 (without blocking reagent), filters were equilibrated in buffer 2 (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 min and transferred in NBT-BCIP solution. Reactions were blocked in 20 mM EDTA.

4.6. In situ hybridization

Antisense digoxigenin(DIG)-labeled riboprobe for *erbB3* was produced from the murine cDNA pDR3 plasmid (a gift from C. Birchmeier) using a DIG-RNA labeling kit (Boehringer), following the manufacturer's instructions. Antisense digoxigenin(DIG)-labeled riboprobe for *HB9* was produced from a murine HB9 cDNA plasmid (a gift from S. Pfaff). Embryoid bodies at day 7 (EB7) were fixed, sectioned to 20 μm on a cryostat, and treated for in situ hybridization as described by Schaeren-Wiemers and Gerfin-Moser (1993). Positive controls were performed using cryostat sections of E14.5 mouse embryo at the level of cranial ganglia (for erbB3 probe) and of spinal cord from E12 mouse embryo (for HB9 probe).

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Spinal Cord Neuronal Precursors Generate Multiple Neuronal Phenotypes in Culture

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Neuronal restricted precursors (NRPs) (Mayer-Proschel et al., 1997) can generate multiple neurotransmitter phenotypes during maturation in culture. Undifferentiated E-NCAM$^+$ (embryonic neural cell adhesion molecule) immunoreactive NRPs are mitotically active and electrically immature, and they express only a subset of neuronal markers. Fully mature cells are postmitotic, process-bearing cells that are neurofilament-M and synaptophysin immunoreactive, and they synthesize and respond to different subsets of neurotransmitter molecules. Mature neurons that synthesize and respond to glycine, glutamate, GABA, dopamine, and acetylcholine can be identified by immunocytochemistry, RT-PCR, and calcium imaging in mass cultures. Individual NRPs also generate heterogeneous progeny as assessed by neurotransmitter response and synthesis, demonstrating the multipotent nature of the precursor cells.

Differentiation can be modulated by sonic hedgehog (Shh) and bone morphogenetic protein (BMP)-2/4 molecules. Shh acts as a mitogen and inhibits differentiation (including cholinergic differentiation). BMP-2 and BMP-4, in contrast, inhibit cell division and promote differentiation (including cholinergic differentiation). Thus, a single neuronal precursor cell can differentiate into multiple classes of neurons, and this differentiation can be modulated by environmental signals.

Key words: E-NCAM; spinal cord development; neuroblasts; stem cells; Shh; BMP

Initially homogeneous neuroepithelial (NEP) stem cells (Kalyani et al., 1997) of the embryonic spinal cord are patterned *in vivo* to generate mature neurons, oligodendrocytes, and astrocytes in a characteristic spatial and temporal profile (Hamburger, 1948; Hirano and Goldman, 1988; Nornes and Das, 1974; Phelps et al., 1988). NEP cell differentiation, both *in vitro* and *in vivo*, likely involves a process of restriction in developmental potential (for review, see McKay, 1997; Stemple and Mahanthappa, 1997). *In vitro*, pluripotent NEP cells generate restricted precursor cells, termed neuronal restricted precursors (NRPs) (Mayer-Proschel et al., 1997), and glial restricted precursors (GRPs) (Rao et al., 1998), which subsequently develop into fully differentiated neuron and glial cells, respectively. Similar cells are present *in vivo* (Mayer-Proschel et al., 1997; Rao and Mayer-Proschel, 1997). *In vivo* neurogenesis precedes and overlaps differentiation of oligodendrocytes and astrocytes (Abney et al., 1981; Frederiksen and McKay, 1988; Hirano and Goldman, 1988; Miller et al., 1985), and is modulated by multiple environmental signals.

Immature neuronal cells undergo additional changes to develop into functional neurons that differ in morphology, receptor profile, and neurotransmitter synthesizing abilities (Phelps et al., 1988, 1990; Ray and Gage, 1994; Richards et al., 1995). These properties are thought to be acquired in a sequential manner during the course of development. Likewise, electrical properties seem to be acquired sequentially (Desarmenien et al., 1993; Walton et al., 1993). For example, functional sodium channels appear before either GABA or glutamate receptors, and spinal neurons therefore show spontaneous electrical activity before any response to GABA or glutamate. Furthermore, the initial GABA responses are depolarizing, and thus they differ from adult inhibitory responses; reversal occurs postnatally (Ben-Ari et al., 1989; Zhang et al., 1990). The acquisition of electrical properties has not been correlated with morphological markers of maturation or the ability to synthesize neurotransmitters.

The acquisition of mature neuronal properties is likely modulated by environmental signals (Cattaneo and McKay, 1990; Echelard et al., 1993; Roelink et al., 1994; Liem et al., 1995, 1997; Muhr et al., 1997; Williams et al., 1997). Two molecules that modulate early neuronal differentiation are bone morphogenetic protein (BMP) and sonic hedgehog (Shh). Shh, a protein secreted by the notochord and floor plate, regulates the induction of motoneurons and some classes of ventral interneurons (Yamada et al., 1993; Roelink et al., 1994). BMP proteins are present dorsally and appear to mediate the generation of dorsal phenotypes (Liem et al., 1995, 1997; Mujtaba et al., 1998). The specific effects of Shh and BMP on isolated neural cells at different developmental stages, and the mechanisms by which they act (proliferation, survival, or differentiation), remain to be determined.

Here we have examined the acquisition of phenotypic properties of NRP cells by comparing immunological and physiological properties of cultured immature neuronal precursors and the mature neurons that differentiate from them. We show that individual NRP cells can differentiate into multiple types of neurons

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and that differentiation occurs via a characteristic pattern of development. In addition, we show that differentiation is modulated by Shh and BMP.

MATERIALS AND METHODS

Substrate preparation

Fibronectin (Sigma, St. Louis, MO) was diluted to a concentration of 20 μg/ml in Tissue Culture H_20 (Sigma). Fibronectin solution was applied to tissue culture dishes for a minimum of 4 hr. Laminin (Biomedical Technologies, Stoughton, MA), used at a concentration of 20 μg/ml, was dissolved in Dulbecco's PBS (DPBS) (Life Technologies/BRL, Gaithersburg, MD). To prepare fibronectin–laminin double-coated dishes, laminin (20 μg/ml) was applied to fibronectin-coated dishes, and plates were incubated overnight. Excess laminin was withdrawn, and the plates were rinsed with NEP medium before cells were plated.

Immunopanning of E-NCAM$^+$ cells

Sprague Dawley rat embryos were removed at embryonic day 13.5 (E13.5) and placed in a Petri dish containing DPBS (Life Technologies/BRL). Spinal cords were mechanically dissected from the surrounding connective tissue with sharpened No. 5 forceps. Isolated spinal cords were incubated in 0.05% trypsin solution for 30 min. The trypsin solution was replaced with NEP medium. The segments were gently triturated with a Pasteur pipette to dissociate cells. E-NCAM$^+$ cells were purified from dissociated cells using a specific antibody-capture assay (Wysocki and Sato, 1978) with minor modifications. In brief, the dissociated cells in suspension were plated on an NCAM antibody (5A5, Developmental Studies Hybridoma Bank)-coated dish to allow binding of all E-NCAM$^+$ cells to the plate. NCAM antibody-coated dishes were prepared by sequentially coating tissue culture dishes with an unlabeled anti-mouse IgM antibody (10 μg/ml) overnight, rinsing dishes with DPBS, followed by coating with 5A5 hybridoma supernatant for 1–3 hr at room temperature. Cells were allowed to bind to the plate for 1 hr at room temperature. Unbound cells were removed, and the plate was rinsed eight times with DPBS. Bound cells were mechanically scraped off and plated on fibronectin–laminin-coated dishes in 1 ml of NEP medium in either mass (5000 cells/dish) or clonal culture (100 cells/dish). Growth factors were added every other day. In all cases, an aliquot of cells was analyzed the next day to determine the efficiency of the immunopanning. In general, >90% of the bound cells expressed detectable NCAM immunoreactivity. Cell populations that did not were repanned or discarded.

NRP cell cultures

Immunopanned E-NCAM$^+$ cells were plated in culture dishes (Corning, Corning, NY) coated with fibronectin–laminin at a dilution of 5000 cells/dish. Cells were maintained at 37°C in 5% $C0_2$/95% air. The basal medium (NEP medium) used in all experiments was a chemically defined medium as described previously (Mayer-Proschel et al., 1997). The medium consisted of DMEM-F12 (Life Technologies/BRL) supplemented with additives described by Bottenstein and Sato (1979) and basic fibroblast growth factor (bFGF) (20 ng/ml).

Clonal cultures of NRP cells

Immunopanned cells were plated in culture dishes (Corning) coated with fibronectin–laminin at a dilution of 100 cells/dish. Approximately 10–20% of single cells died within 24 hr. The remaining single cells were allowed to proliferate until large enough to assay (between 7 and 10 d). Clones were isolated using glass cloning rings. Greased rings were placed around a selected, well isolated clone, and cells were dissociated from the substrate using trypsin-EDTA. Dissociated cells were aspirated, and the trypsin-EDTA was neutralized with excess medium. For RT-PCR analysis, clonal cells were then centrifuged and harvested for RNA isolation. For calcium imaging experiments, clones were replated on poly-lysine/laminin-coated glass coverslips. Cells were imaged 24–48 hr after replating to ensure that they had recovered from the replating process. For immunocytochemistry, cells were replated on poly-lysine/laminin-coated dishes and allowed to settle and extend processes before being analyzed. In all cases, clones were grown in NEP medium as described above [also see Kalyani et al. (1997)], supplemented with neurotrophin-3 (10 ng/ml).

Differentiation of NRP cells and Shh and BMP treatment

Neuronal differentiation of cultured NRP cells was induced by plating on poly-lysine/laminin-coated dishes (poly-lysine at 20 μg/ml in DPBS; Sigma), and reducing FGF concentration with the addition of RA (1 μM). In some experiments, neuronal differentiation was induced by the addition of BMP-2 or BMP-4 at 10 ng/ml. The effects of Shh and BMP were assessed by supplementing NEP medium with Shh at doses of 1–100 ng/ml, or BMP-2 or BMP-4 at 10 ng/ml. To assess the effect of Shh on mitosis, we withdrew FGF from cultures, rinsed them with FGF-free

Table 1. Primer sequences used for PCR amplification

Gene	Product size (bp)	Primers (sense, antisense)
p75	329	5′-GCA CAT ACT CAG ACG AAG CCA-3′ 5′-AGC AGC CAA GAT GGA GCA ATA GAC-3′
ChAT	377	5′-CTG AAT ACT GGC TGA ATG ACA TG-3′ 5′-AAA TTA ATG ACA ACA TCC AAG AC-3′
Isl-1	350	5′-GCA GCA TAG GCTTCA GCA AG-3′ 5′-GTA GCA GGT CCG CAA GGT G-3′
GAD_{65}	327	5′-GAA TCT TTT CTC CTG GTG GTG-3′ 5′-GAT CAA AAG CCC CGT ACA CAG-3′
Calbindin28	276	5′-GCA GAA TCC CAC CTG CAG-3′ 5′-GTT GCT GGC ATC GAA AGA G-3′
Glutaminase	560	5′-GCA CAG ACA TGG TTG GGA TAC TAG-3′ 5′-GCA GGG CTG TTC TGG AGT CG-3′
Smoothened	467	5′-GGA CAG ACA ACC CCA AGA GC-3′ 5′-CCG AGA GAG GCT GGT AAG TGG-3′
Patched	1297	5′-CAA TGG CTA CCC CTT CCT GTT CTG-3′ 5′-CGG CCT CTC CTC ACA TTC CAC-3′
glyRa2	330	5′-CAG AGT TCA GGT TCC AGG G-3′ 5′-TCC ACA AAC TTC TTC TTG ATA G-3′
$GABA_A$-R	589	5′-TGG AC(TC) CC(AT) GA(TC) AC(ACT)TT(TC) TT-3′ 5′-GC(AGTC) AT(GA) AAC CA(GA)TCC ATG GC-3′
Cyclophilin	302	5′-CCA CCG TGT TCT TCG ACA TC-3′ 5′-GGT CCA GCA TTT GCC ATG G-3′

medium, and added Shh (100 ng/ml) in the presence of the survival agent NT-3. This was required, because quantifying an increase in mitosis in the presence of saturating concentrations of FGF was technically difficult. For all other experiments, the effects of Shh with or without FGF were identical. BMP-2, -4, and -7 were obtained from Genetics Institute (Cambridge, MA), and Shh (N-terminal form) was obtained from Ontogeny Inc.

Gene detection by RT-PCR

RNA isolation. Total RNA was isolated from cells or whole tissues by a modification of the guanidine isothiocyanate-phenol-chloroform extraction method (Trizol, Life Technologies/BRL).

cDNA synthesis. cDNA was synthesized using 1–5 μg of total RNA in a 20 μl reaction. Superscript II (Life Technologies/BRL), a modified Maloney murine leukemia virus reverse transcriptase, and oligo-dT_{12-18} primers were used, and the Life Technologies/BRL protocol was followed.

PCR. Aliquots of cDNA, equivalent to one-twentieth of the above reaction, were used in a 50 μl reaction volume. PCR amplification was performed using ELongase polymerase (Life Technologies/BRL). Primer sequences used for PCR amplification are shown in Table 1. In general, the reactions were run for 35 cycles, unless stated otherwise, and a 10 min incubation at 72° was added at the end to ensure complete extension. In the case of GABA receptors, co-amplification of $GABA_A$R-subunits was performed using degenerate primers for the α-subfamily (subunits 1–6) (Berger et al., 1998). The PCR products were purified using the Advantage PCR-Pure Kit (Clontech, Palo Alto, CA) and sequenced to confirm their identities. All PCR results reported were reproduced reliably using cDNA collected from five to seven independent experiments unless stated otherwise.

Quantification of ptc *relative levels.* The same amount of cDNA was used in PCR reactions using primers for *cyclophilin* and *ptc*. PCR with cyclophilin primers was performed for 23 cycles, and PCR with ptc primers was preformed for 35 cycles; amplification was within the linear range. The ratio of *cyclophilin* to *ptc* within a sample was determined by scintillation radiometry (ImageQuant, Molecular Dynamics, Sunnyvale, CA). This ratio was then compared across samples within each experiment, and the SEM was determined.

Immunocytochemistry

Staining procedures were as described previously (Rao and Mayer-Proschel, 1997). Staining for the cell surface markers p75 and E-NCAM was performed in cultures of living cells. To stain cells with antibodies against internal antigens, cultures were fixed with 2–4% formaldehyde for 30 min at room temperature. In general, dishes were incubated with the primary antibody for 1 hr followed by incubation with an appropriate secondary antibody for 1 additional hr. The peroxidase reaction for HRP-conjugated secondaries was performed as described previously (Rao et al., 1992). Double-labeling experiments were performed by simultaneously incubating cells in appropriate combinations of primary antibodies followed by noncross-reactive secondary antibodies. Diamino-bisbenzimide (DAPI) (Signal) histochemistry was performed as described previously (Kalyani et al., 1997). DAPI staining was generally performed after all other antibody staining had been completed.

p75, E-NCAM, and nestin monoclonal antibodies (Lendahl et al., 1990; Dahlstrand et al., 1995) were hybridoma supernatants obtained from the Developmental Studies Hybridoma Bank (DSHB). β-III tubulin antibody, which stains neurons, was obtained from Sigma. An anti-nestin polyclonal antibody used in some double-labeling experiments was the kind gift of Dr. Keith Cauley (Signal Pharmaceuticals). Antibodies to choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), and glutamic acid decarboxylase (GAD) were obtained from Chemicon (Temecula, CA) and used at dilutions recommended in the enclosed protocols. Antibodies to glutamate and glycine were obtained from Signature Immunologicals and used according to manufacturer's recommendations. 5-Bromodeoxyuridine (BRDU, Sigma) was used to determine the number of dividing cells. Mouse and rat monoclonal anti-BRDU antibodies were obtained from Boehringer Mannheim (Indianapolis, IN). All secondary monoclonal antibodies were purchased from either Jackson ImmunoResearch (West Grove, PA) or Southern Biotechnology (Birmingham, AL).

Neutralizing antibody experiments

E-NCAM$^+$-immunopanned cells were plated on fibronectin–laminin-coated dishes in basal medium supplemented with NT-3. Four hours after plating, cells were treated with Shh (100 ng/ml), neutralizing anti-Shh hybridoma supplement (50 μl/ml), or control (anti-A2B5) hybridoma supernatant. Cells were grown in combinations of Shh and antibody as described in Results for a period of 48 hr. Cultures were pulsed with BRDU in the last 3 hr of culture, fixed, and processed for BRDU incorporation and DAPI histochemistry as previously described. BRDU-incorporating cells were counted in five randomly chosen fields, and the number of dividing cells was expressed as a percentage of the DAPI-labeled nuclei in the same fields. All experiments were performed in duplicate, and the experiments were repeated at least twice. Results are presented as the mean of two experiments ± SEM.

Intracellular calcium measurements

Ca^{2+} imaging experiments were performed on both mass and clonal cultures of E-NCAM$^+$ cells obtained as described above. Cells were loaded with 5 μM fura-2 AM (Grynkiewicz et al., 1985) plus pluronic F127 (80 μg/ml) in rat Ringers's solution (RR) at 23°C in the dark for 20 min followed by three washes in RR and a 30 min de-esterification. Relative changes in intracellular $[Ca^{2+}]$ were measured from the background-corrected ratio of fluorescence intensity by excitation at 340/380 nm. Response was defined as a minimum rise of 10% of the ratioed baseline value. A Zeiss-Attofluor imaging system and software (Atto Instruments, Rockville, MD) were used to acquire and analyze the data. Data points were sampled at 1 Hz. Neurotransmitters were made in RR and delivered by bath exchange using a small volume loop injector (200 μl). RR consisted of (in mM): 140 NaCl, 3 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose. Ascorbic acid (500 μM) was added to dopamine solutions to prevent oxidation. Control application of 500 μM ascorbic acid had no effect (data not shown). The pH of all solutions was adjusted to 7.4 with NaOH. K^+ RR (50 mM) was made by substituting equimolar K^+ for Na^+ in the normal RR.

RESULTS

E-NCAM$^+$ cells mature in culture

We have previously identified E-NCAM$^+$-expressing cells of E13.5 neural tube as neuronal restricted precursors (Mayer-Proschel et al., 1997). Immunocytochemical studies have shown that virtually 100% of the E-NCAM$^+$ cells dissociated at this time express the early neuronal marker microtubule-associated protein kinase-2 (MAP-2 kinase), ~50% express βIII-tubulin, but only ~10% express the neuronal marker NF-M when cultured in nondifferentiation promoting medium that includes fibroblast growth factor (FGF) as a survival agent (Mayer-Proschel et al., 1997). After the removal of FGF and the addition of retinoic acid (RA), E13.5 E-NCAM$^+$ cells exit the mitotic cycle, increase in soma size, and begin to extend extensive processes.

To determine more accurately the process of *in vitro* maturation, we performed several experiments that were repeated between six and nine times with similar and reproducible results. The proportion of dividing cells was compared in acutely dissociated and differentiated cells using BRDU incorporation. Approximately 70% of acutely dissociated cells incorporate BRDU (Fig. 1*A*), whereas after 10 d in culture (in differentiation-promoting medium), few or no cells incorporate BRDU and therefore had stopped dividing (Fig. 1*B*). Next, we examined the acquisition of NF-M, which is not expressed by the majority (~90%) of E13.5 E-NCAM$^+$ NRP cells (Mayer-Proschel et al., 1997), by immunocytochemistry. Cells were double-labeled with NCAM (Fig. 1*C,D*) and NF-M (Fig. 1*c,d*). Figure 1*c,d* shows that very few acutely dissociated cells express NF-M, whereas nearly all differentiated cells express the protein. Similarly, synaptophysin, a protein specifically associated with synaptic vesicles and functional synapses (for review, see Sudhof, 1995), is expressed by differentiated (Fig. 1*f*) but not acutely dissociated E-NCAM$^+$ cells (Fig. 1*e*). Although synaptophysin protein expression is associated with synaptic vesicles, early expression (as seen in Fig. 1*f*) can be detected in the cell bodies and throughout the lengths

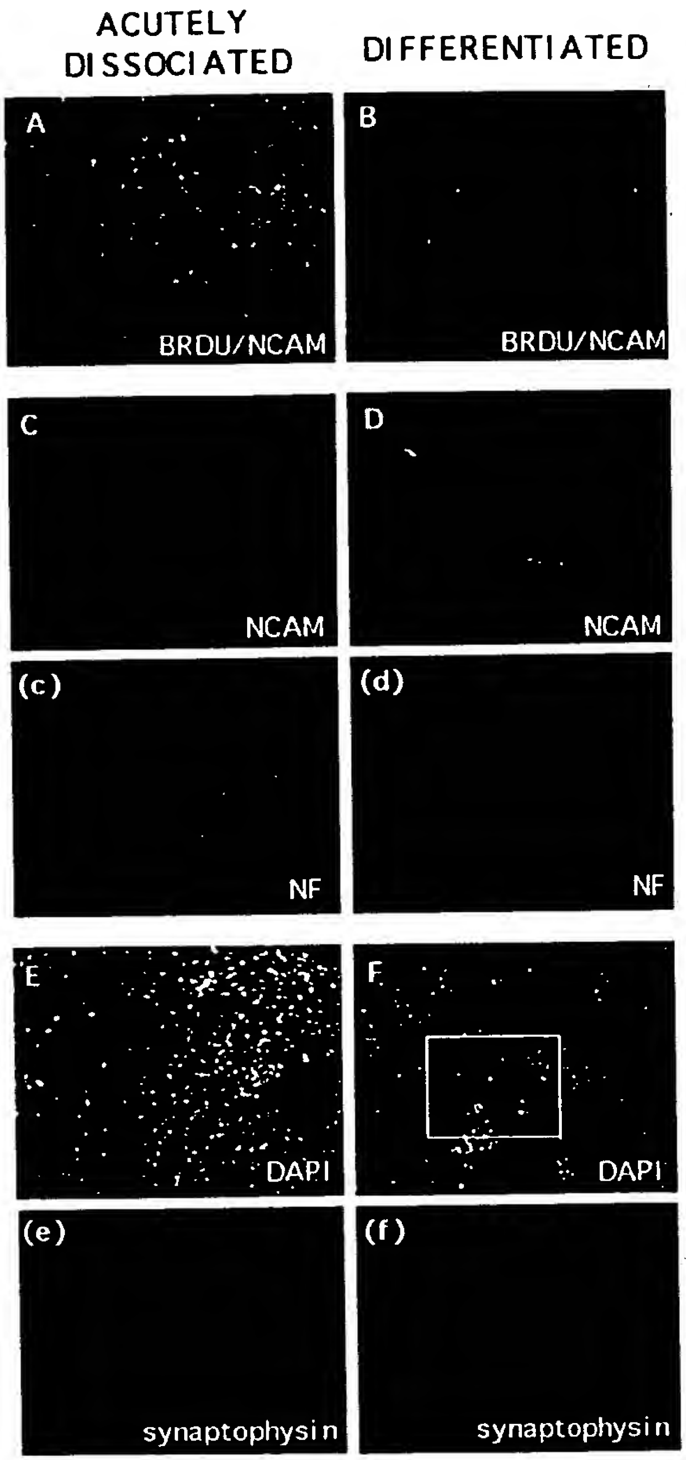

Figure 1. Differentiated E-NCAM$^+$ cells can be distinguished from acutely dissociated NRP cells. E-NCAM$^+$ cells were isolated by immunopanning, plated in 35 mm dishes, and allowed to grow for 24 hr (acutely dissociated) or for 10 d (differentiated). Cultured cells were then analyzed for cell division, E-NCAM expression, NF-M expression, and synaptophysin expression. Most acutely dissociated E-NCAM$^+$ cells (*green, A*) incorporate BRDU (*red, A*). In contrast, few differentiated E-NCAM$^+$ cells (*green, B*) are BRDU immunoreactive (*red, B*). Most acutely dissociated E-NCAM$^+$ cells (*green, C*) were not NF-M immunoreactive (*red, c*). In contrast, all differentiated E-NCAM$^+$ cells (*green, D*) express NF-M (*red, d*). In addition, acutely dissociated cells do not express synaptophysin as assessed by synaptophysin staining (*green*) and DAPI (compare *e* and *E*, respectively). A subset of differentiated E-NCAM$^+$ cells, however, have begun to express detectable levels of synaptophysin (compare *f* and *F*, respectively; *f* is a magnification of the area within the *white box* in *F*). Thus, acutely dissociated E-NCAM$^+$ cells are immature, dividing cells that mature in culture. Objective magnification, 20×.

of the processes where it is initially expressed during early neurogenesis (Grabs et al., 1994; Fujita et al., 1996). Corresponding nuclear DAPI staining is shown in Figure 1*E,F* to demonstrate the location of the cell bodies. These results suggest that if NRP cells are induced to differentiate by RA and the removal of mitogen, they acquire many morphological and immunological properties of mature neurons.

Cultured E-NCAM$^+$ cells can synthesize and respond to multiple neurotransmitters

We have shown previously that NRP cells can differentiate into postmitotic neurons but not into oligodendrocytes or astrocytes (Mayer-Proschel et al., 1997). To determine whether NRPs can differentiate into all of the major neuronal phenotypes present in the spinal cord, or whether they are more limited in their differentiation potential, we examined the expression of neurotransmitter synthesizing enzymes and cell type-specific markers for mature neurons after inducing NRPs to differentiate.

E-NCAM$^+$ cells from E13.5 rat neural tube were isolated by immunopanning and cultured in differentiation-promoting medium (see Materials and Methods). After 10 d in culture, we analyzed the synthesis of the neurotransmitters acetylcholine (ACh), GABA, and glutamate (as assessed by the expression of their synthesizing enzymes) using RT-PCR (Table 1). In addition, we examined the expression of p75 (Yan and Johnson, 1988) and Islet-1 (Isl-1) (Tsuchida et al., 1994), which are characteristic of motoneurons in the spinal cord, and calbindin, which is often co-expressed with GABA (Batini, 1990). As shown in Figure 2, Panel 1, all of these were present in differentiated cells (labeled *D* in Panel 1). In contrast, none of these markers of neurotransmitter phenotypes could be detected from cells that were examined within 24 hr of isolation (termed "acutely dissociated"; labeled *AD* in Panel 1), although we could readily detect the housekeeping gene *cyclophilin* from both cell populations (as shown in Panel 1). Thus, our data show that neuron-restricted precursor cells mature in culture and that NCAM expression and neuronal fate determination precede neurotransmitter synthesis.

We also examined the expression of neurotransmitters or their synthesizing enzymes by immunocytochemistry to determine whether all cells or only subsets of differentiated cells express these markers. Cells were grown in culture for 10 d and allowed to differentiate and then were fixed and processed by immunocytochemistry to detect expression of ChAT (Fig. 2, *Panel 2, B*), GAD (*D*), TH (*F*), glycine (*H*), and glutamate (*J*). Virtually 100% of the differentiated cells expressed detectable glutamate levels, and this percentage did not vary significantly from experiment to experiment (n = 6). A smaller percentage expressed glycine and GAD. Percentages varied between experiments from 10 to 50%. The percentages of ChAT and TH$^+$ cells ranged between 1 and 5% (these cells usually appeared in clumps, rather than being equally distributed throughout the population, as seen in Fig. 2, *Panel 2, B,F*). The use of nonspecific differentiation-promoting factors resulted in considerable variability in the numbers of any given phenotype generated (except glutamate) from experiment to experiment (n = 6 independent experiments). However, although the exact percentages varied, heterogeneity of

Table 2. Acutely dissociated and differentiated E-NCAM$^+$ cells differ in their properties

	Acutely dissociated	Differentiated
Mitotic status	Mitotic	Postmitotic
Cell size	Comparatively smaller	Comparatively larger
Process outgrowth	Little or none	Extensive
Neuronal markers	NCAM βIII-tubulin MAP-2 kinase Nestin	NCAM βIII-tubulin MAP-2 kinase NF-M Synaptophysin Peripherin
Neurotransmitters, neurotransmitter synthetic enzymes, or other phenotype-specific markers	None, except for a small amount of glutamate immunoreactivity	Glutamate Glycine Glutaminase Glutamic acid decarboxylase Choline acetyltransferase Islet-1 p75 Calbindin
Response to neurotransmitters	Weak and in a small subset of cells	Robust and in virtually all cells
Depolarizing response to GABA and glycine	Numerous cells with depolarizing responses detected	Few or no cells with depolarizing responses detected

The differences in the morphological and immunocytochemical properties as well as differences in response to neurotransmitter application are summarized. Acutely dissociated cells overall were immature and expressed few neuron-specific markers. Maturation occurred in culture, and cells grown in culture appeared similar to cells isolated from early postnatal spinal cord (see Discussion). Note that only depolarizing responses to GABA and glycine could be assessed by fura-2 Ca^{2+} imaging. Cells listed as not responding may have had hyperpolarizing responses.

phenotypes did not, and in all cases significant numbers of all phenotypes were detected. Also, because virtually 100% of the cells synthesize glutamate, it is likely that at least some cells synthesize more than one neurotransmitter. Nevertheless, these results clearly demonstrate that during differentiation, E-NCAM$^+$ cells are capable of maturing into a heterogeneous population with respect to their neurotransmitter phenotype.

In contrast to the results obtained with differentiated cells, neither ChAT, GAD, TH, nor glycine could be detected in acutely dissociated cells. We did detect glutamate in a small subset of cells (<10%; data not shown) from each experiment ($n = 6$). We were unable, however, to detect glutaminase in these cells by RT-PCR (Fig. 2, *Panel 1*), which may suggest that glutamate is being taken up by these cells from the medium.

Another important characteristic of mature neurons is their ability to respond to multiple neurotransmitters by expressing appropriate neurotransmitter receptors on their surface. To examine the ability of differentiated E-NCAM$^+$ cells to respond to glutamate, glycine, dopamine, and acetylcholine, we used fura-2 Ca^{2+} imaging techniques. E13.5 E-NCAM$^+$ cells were grown in culture for 10 d and allowed to differentiate. They were then loaded with fura-2 (see Materials and Methods), and the depolarizing response to neurotransmitter application was monitored. Figure 3*A* shows a bar graph of the number of cells (sum of cells from three independent experiments) responding to application of the indicated neurotransmitter on acutely dissociated and differentiated cells. In general, the number of cells responding to neurotransmitters and the amplitude of the neurotransmitter-induced Ca^{2+} responses increased in the differentiated cells. The most striking example was dopamine, where only 10% of acutely dissociated cells responded to 500 μM dopamine with increases in internal Ca^{2+} compared with 76% of differentiated cells, a net increase of 66%. Similar but less striking changes in the number of cells responding were seen for other excitatory neurotransmitters (Fig. 3*A*). The exceptions to this trend were the Ca^{2+} responses to GABA and glycine. Interestingly, 47% of the acutely dissociated cells responded to GABA compared with only 8% of the differentiated cells. Similarly, Ca^{2+} flux in response to glycine decreased from 20% in the acutely dissociated cells to 0% in the differentiated cells. To rule out the possibility that the cells were downregulating their receptors for these transmitters as they matured, we checked for the expression of GABA$_A$-α1–6 receptor subunits (Berger et al., 1998) and GlyR-α2, the predominant embryonic glycine receptor subunit expressed in neurons throughout the CNS (Heck et al., 1997). Both of these were readily detected by RT-PCR from cDNA of mature NCAM$^+$ cells (data not shown). Therefore, the change in the inhibitory neurotransmitter profile probably reflects the decrease in internal Cl^- concentration with maturation that accounts for the shift from depolarizing to hyperpolarizing GABA and glycine responses (Wu et al., 1992).

Representative plots of ratioed (I_{340}/I_{380}) Ca^{2+} responses over time from an acutely dissociated and differentiated cell are shown in Figure 3, *B* and *C*, respectively. Figure 3*D* shows images of the cells from which the data were taken; the cell represented in the plot is indicated by a letter in the image. The acutely dissociated cell responded to GABA and glutamate, whereas the differentiated cell from the same embryo responded to dopamine, glutamate, and acetylcholine but not to GABA or glycine. Comparison of Ca^{2+} responses to the various transmitters in adjacent cells reveals that there is heterogeneity in the response profiles among cells, indicating that not only are the E-NCAM$^+$ cells heterogeneous in their ability to synthesize neurotransmitters, they are also selective in terms of transmitter receptor expression. In addition to neurotransmitters, elevated K^+ in RR (50 mM K^+ RR) was applied to depolarize the cells and allow Ca^{2+} entry through voltage-gated channels. In acutely dissociated cells, 49% responded to 50 mM K^+ RR compared with 85% of differentiated

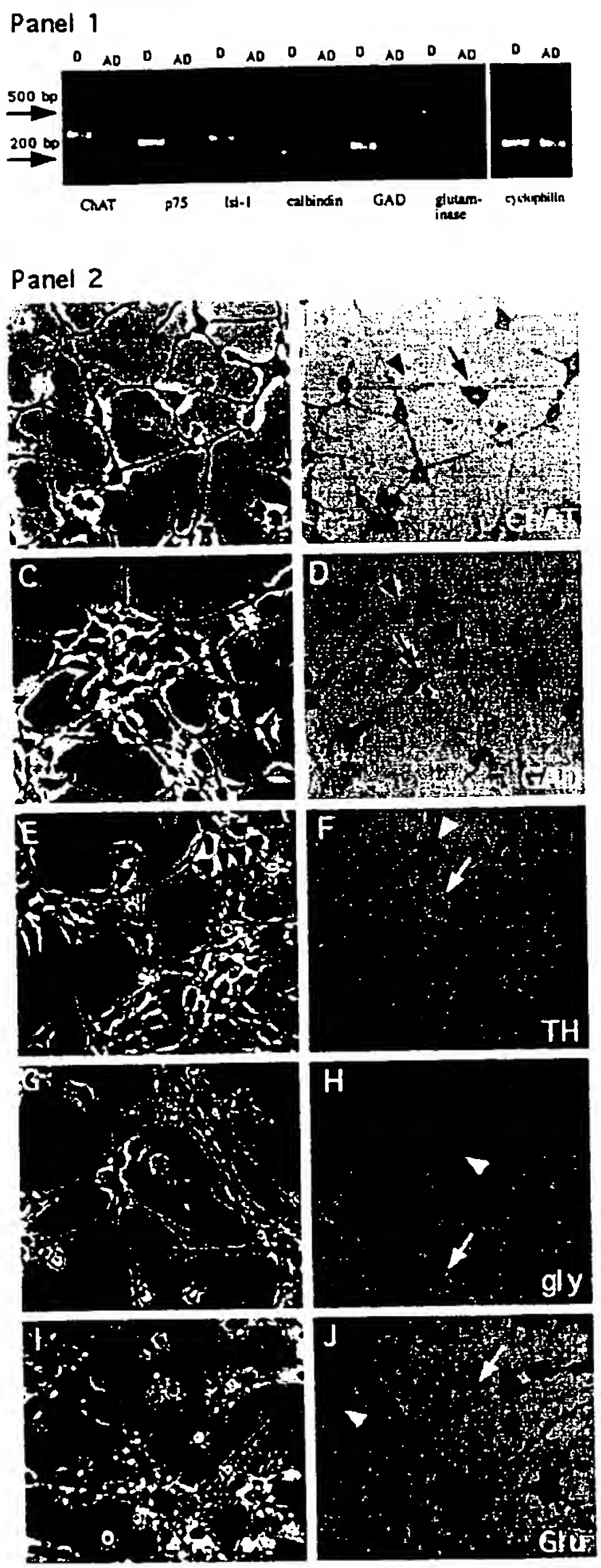

Figure 2. Numerous neuronal phenotypes can be detected by RT-PCR and antibody staining in differentiated but not acutely dissociated E-NCAM$^+$ cells. E-NCAM$^+$ cells were isolated by immunopanning, plated in 35 mm dishes, and allowed to grow for 24 hr (acutely dissociated), or for 10 d (differentiated). Cultured cells were then analyzed by RT-PCR (*Panel 1*) or by immunocytochemistry (*Panel 2*). *Panel 1*, RNA was isolated from acutely dissociated (*AD*) and differentiated E-NCAM$^+$ cells (*D*) and assayed for the expression of ChAT, p75, Isl-1, calbindin, GAD, glutaminase, and cyclophilin (a housekeeping gene) by RT-PCR. Acutely dissociated cells did not express any of the genes tested except cyclophilin. In contrast, all markers were readily detected in differentiated cells. Thus, E-NCAM$^+$ cells begin to express neurotransmitter-synthesizing enzymes or phenotypic markers after differentiation in culture. *Panel 2*, Differentiated E-NCAM$^+$ cells were fixed and stained with antibodies to ChAT (*A, B*), GAD (*C, D*), TH (*E, F*), Gly (*G, H*), and Glu (*I, J*). Phase (*A, C, E, G, I*) and bright-field (*B, D, F, H, J*) images of representative fields showing staining with each antibody are shown. Note that subsets of E-NCAM$^+$ neurons expressed each marker, the proportions of which differed between antibodies [note both the presence (*arrow*) and absence (*arrowhead*) of staining in different cells of the same population]. Objective magnification, 20×.

cells (Fig. 3), suggesting that more of the differentiated cells were electrically competent than were the acutely dissociated cells.

Thus, the contrast between various properties of acutely dissociated E-NCAM$^+$ cells and fully differentiated E-NCAM$^+$ cells, which is summarized in Table 2, is striking. Immature cells are mitotically active, and differentiated cells are not; immature cells do not express neuronal proteins such as NF-M, synaptophysin, or neurotransmitter synthetic enzymes, whereas all of these can be detected in differentiated cells; and acutely dissociated cells are overall less responsive than differentiated cells to neurotransmitter-induced Ca^{2+} responses.

Individual E-NCAM$^+$ cells can generate multiple neurotransmitter phenotypes

Our mass culture experiments show that the E-NCAM$^+$ population can generate multiple neurotransmitter phenotypes. However, there exists the possibility that individual cells are precommitted to generating specific neuronal phenotypes. To determine whether the differentiation potential of NRPs in mass culture reflected the potential of an individual NRP, we performed clonal analysis of E-NCAM$^+$ cells. E-NCAM$^+$ cells were immunoselected, plated at clonal density, and grown in FGF and NT-3, conditions that promote proliferation. Clones grew to sizes of several hundred cells after 7–10 d in culture, after which their differentiation was promoted by withdrawal of FGF and addition of RA in the medium.

Three different techniques were used to determine whether clones generated from individual NRP cells were composed of heterogeneous populations of neurons: RT-PCR, immunocytochemistry, and calcium imaging. Six clones were examined by RT-PCR analysis. Of these clones, 5/6 expressed multiple neurotransmitter phenotypes (Fig. 4*A*); one clone expressed all six markers tested, 3/6 expressed four markers, and 1/6 expressed three markers. Therefore, all but one clone were composed of heterogeneous populations of cells. One clone expressed detectable levels of only p75 and Isl-1 but not ChAT. This likely represents an immature clone that we had not allowed to fully differentiate. These observations that individual clones express multiple neurotransmitter synthetic enzymes or other phenotypic markers indicates that most clones were composed of a heterogeneous population.

To confirm the PCR results and to show heterogeneity at the protein level, clones were analyzed for the presence of p75 expression. No clone (0/17) consisted exclusively of p75-immunoreactive cells, but all clones (17/17) contained

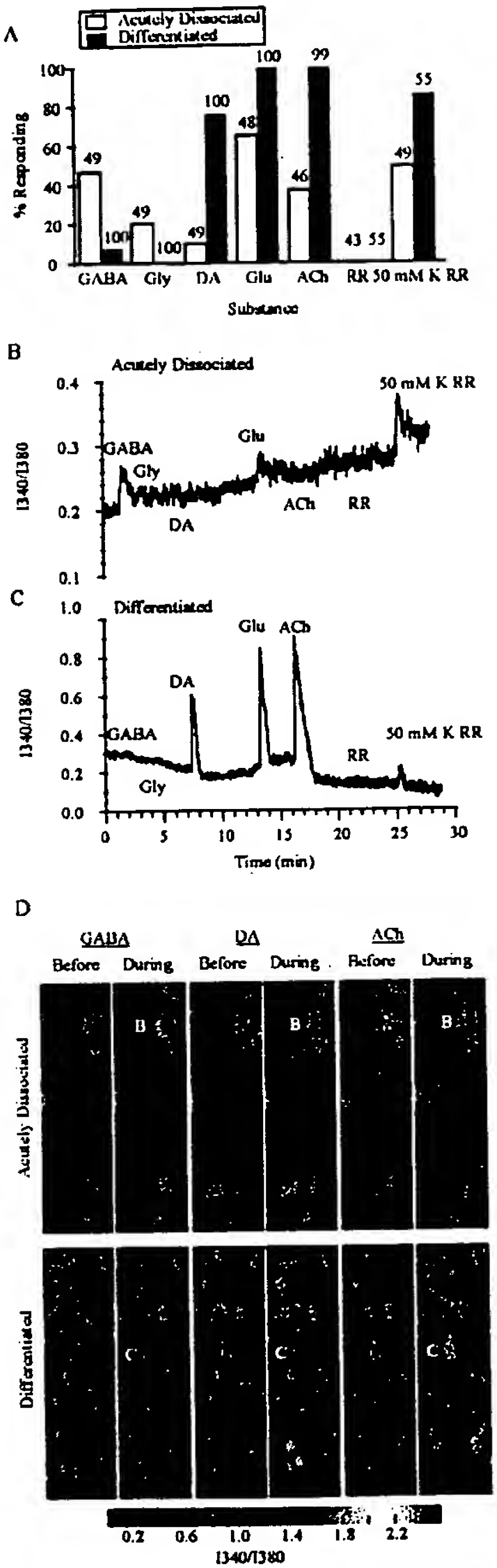

Figure 3. The neurotransmitter receptor profile of E-NCAM$^+$ cells changes with maturation. Changes in $[Ca^{2+}]_i$ were recorded from acutely isolated and differentiated E-NCAM$^+$ cells loaded with the Ca^{2+} indicator dye, fura-2. *A*, The *bar graph* shows the percentage of E-NCAM$^+$ cells that responded to 500 μM GABA, glycine (*Gly*), dopamine (*DA*), glutamate (*Glu*), acetylcholine (*ACh*), rat Ringer's solution control (*RR*), and elevated K^+ (50 mM K^+ RR). The response profile changed for acutely dissociated cells (*open bars*) compared with differentiated cells (*closed bars*; number of cells measured is indicated above each bar). The percentage of differentiated cells responding was significantly different from acutely isolated cells with all test substances (Student's *t* test, $p < 0.0001$). *B, C*, The ratio of intensity from excitation at 340 nm and 380 nm plotted over time shows relative changes in $[Ca^{2+}]_i$ from acutely dissociated (*B*) and differentiated cells (*C*) in response to neurotransmitters, RR control, and 50 mM K^+. Note the difference in scale for *B* and *C*. *D*, Images of E-NCAM$^+$ cells during responses to neurotransmitters. The *left half* of each image is from before neurotransmitter application, and the *right half* is during the response. The *traces* in *B* and *C* were taken from the cells indicated with *letters* in *D*.

p75-immunoreactive cells as well as other neurons. An example of a clone showing a subset of cells expressing p75 is shown in Figure 4*B*. Similarly, staining with either glutamate or glycine showed that each transmitter is expressed by only a subset of cells in the same clonal population, indicating that clones are a heterogeneous population. Figure 4*C* shows cells from the same clone, which was split and plated into two separate dishes. One dish was stained with glutamate, and the other dish was stained with glycine. As with the E-NCAM$^+$ cells in mass culture, nearly 100% of the clonal cells were immunoreactive for glutamate, and a smaller percentage (10–50%; $n = 17$) was immunoreactive for glycine. These results provide further evidence that E-NCAM$^+$ cells generate multiple neuronal subtypes in culture.

Heterogeneity can be demonstrated not only by the synthesis of different neurotransmitters, but also by heterogeneity in the receptors expressed by the cells. We examined response profiles of differentiated clonal cells to application of GABA, glycine, dopamine, glutamate, acetylcholine, and 50 mM K^+ RR, as evidenced by increased intracellular calcium concentrations. Ca^{2+} measurements were taken from as many as 113 cells from four different clones. All clones examined (four of four) displayed heterogeneity in their response profiles, which varied somewhat between individual clones (data not shown). Figure 5*A* shows a bar graph of the percentage of cells from all four clones that responded to each of the applied neurotransmitters.

As with the mass cultures of differentiated E-NCAM$^+$ cells, high percentages of differentiated clonal cells responded to glutamate (93%), acetylcholine (96%), 50 mM K^+ RR (70%), and dopamine (50%), whereas few cells responded to GABA (27%) and glycine (1%). Figure 5*B,C* shows representative traces of the ratio (I_{340}/I_{380}) of Ca^{2+} responses from two cells recorded from one clone. Images of these cells and the heterogeneity of receptor profiles within the clonal population are shown in Figure 5*D*. Of the cells shown in Figure 5*D*, one responded to dopamine (labeled *C*; trace shown in Fig. 5*C*), whereas a nearby cell (labeled *B*; trace shown in Fig. 5*B*) did not, suggesting that they expressed different receptors. This heterogeneous expression of receptors also indicates the multipotential characteristic of individual NRP cells. Thus, the maturation of clonal populations of cells closely resembles the maturation of cells in mass culture.

By multiple independent methods, our clonal analysis demonstrates the multipotential characteristic of individual NRP cells. This analysis confirms our mass culture results, which clearly define the developmental potential of the NRP cell. Although committed to generating neurons, the particular phenotypes of its progeny are dictated at some later stage in their development. Thus, we have established the existence of a neuronal precursor cell that can be purified and subsequently manipulated to define the transition between lineage-restricted neuronal precursor and differentiated neuronal progeny.

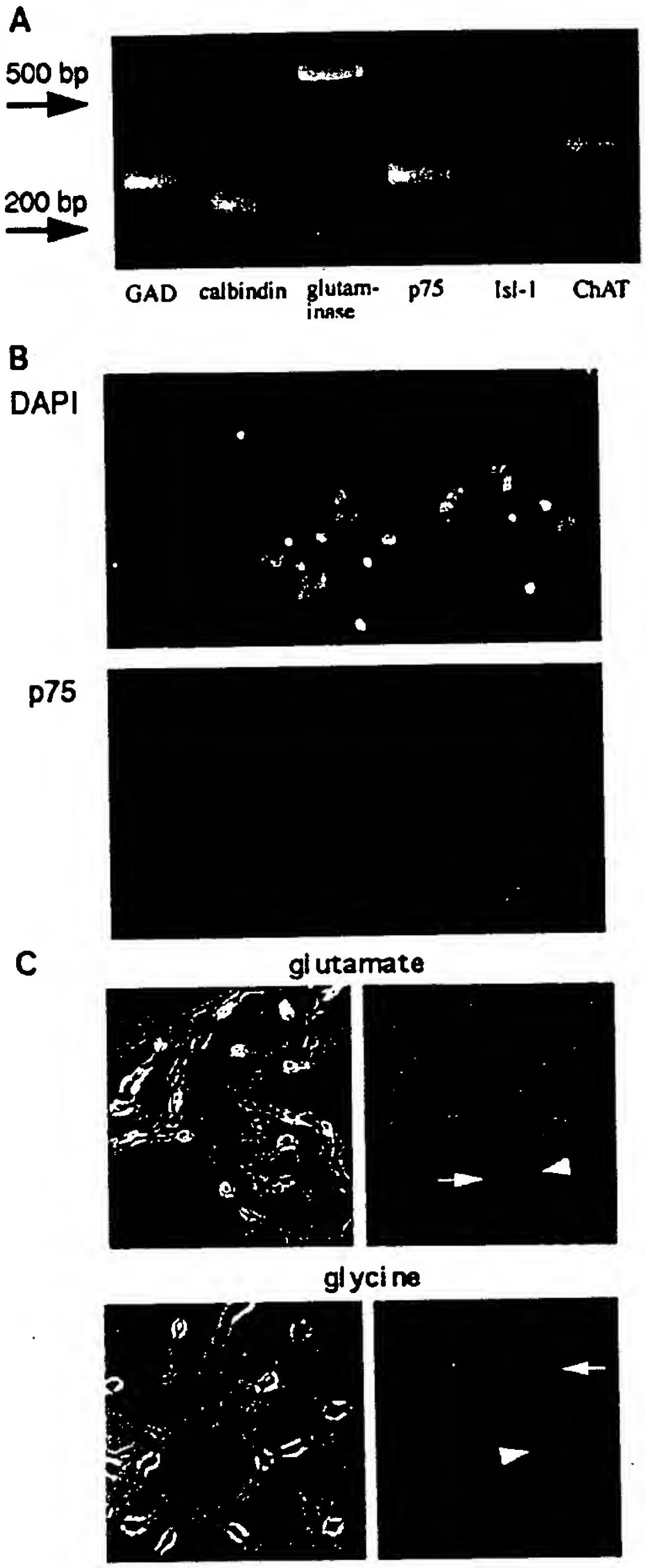

Figure 4. Individual E-NCAM$^+$ cells can generate multiple neurotransmitter phenotypes. E-NCAM$^+$ cells were plated at clonal density, and individual clones were followed and allowed to grow for a period of 7–10 d. Clones were then analyzed by RT-PCR (*A*) or by immunocytochemistry (*B, C*). *A*, RNA was isolated from individual E-NCAM$^+$ clones and assayed for the expression of GAD, calbindin, glutaminase, p75, Isl-1, and ChAT by RT-PCR. This clone expressed all of the markers tested ← *B*, E-NCAM$^+$ clones were immunostained with p75, a marker for motoneurons (see Results) and counterstained with DAPI to identify all cells. *B* shows a representative clone in which a subset of cells were immunoreactive for p75, demonstrating the heterogeneity of the progeny of a single E-NCAM$^+$ cell. *C*, A representative E-NCAM$^+$ clone that was split into two different dishes. One dish was stained with glutamate and the other with glycine. Note that although the majority of progeny from a single progenitor are immunoreactive for glutamate, significantly less are immunoreactive for glycine, indicating a heterogeneous clonal population. Objective magnification, 20×.

Extracellular signals influence the fate of NRP cells

Our results show that neuronal precursors can develop *in vitro* into mature neurons of multiple phenotypes in both mass and clonal cultures and that either application of RA or removal of FGF can promote differentiation into multiple phenotypes. In normal development, however, differentiation is spatially and temporally regulated, with motoneurons being generated ventrally and sensory neurons being generated dorsally, suggesting that specific environmental signals may bias differentiation of neuronal precursors (Ray and Gage, 1994; Richards et al., 1995). We examined the effects of two potential regulatory molecules that are expressed in the spinal cord at the time of neurogenesis and have been shown to bias cells to either dorsal (for review, see Graff, 1997) or ventral (for review, see Fietz et al., 1994) phenotypes, BMP-2/4 and Shh, respectively.

When BMP-2 was added to cultures of E-NCAM$^+$ cells, a dramatic reduction in cell division was seen. The effect of BMP-2 overrode the effect of the mitogen FGF; even in the presence of FGF, BMP-2 caused a 60% reduction in cell division (Fig. 6*K*). Identical effects were seen with BMP-4 (data not shown). BMP-2 was not a survival factor, because cells grown in BMP-2 alone did not survive. The decrease in mitosis was accompanied by the appearance of differentiated cells. Cell size increased and cells put out extensive processes (for example, see Fig. 6*F*). Cells grown in BMP-2 for 48 hr were examined for neurotransmitter expression. Glutamatergic, GABAergic, dopaminergic, and cholinergic neurons were detected (data not shown) (Fig. 6*F,I*). The number of cholinergic neurons was significantly larger than in untreated controls (5–10 vs 0–1%) (Fig. 6, compare *F,I* with *D,G*); however, there appeared to be no bias toward ventral phenotypes because the promotion of all other phenotypes was also significantly larger. Thus, BMP-2 acted as an antimitotic agent and promoted differentiation of E-NCAM$^+$ NRP cells but did not appear to inhibit ventral fates.

In contrast to the anti-mitotic and differentiation-promoting effect of BMP-2/4, Shh appeared to be a mitogen. The mitotic effect of Shh at 100 ng/ml (the maximal response) was threefold over controls but was less than the effect of FGF at 10 ng/ml (Fig. 6*J*). Experiments with Shh were performed in the presence of NT-3, which acts as a survival agent (for review, see Barde, 1994), and not as a mitogen, because Shh itself did not appear to be a survival factor for E-NCAM$^+$ cells; E-NCAM$^+$ cells grown in Shh alone did not survive. The effect of Shh on mitosis was only apparent after 2 d of exposure and was maintained over the 5 d of the assay.

To confirm that the effect on mitosis was caused by Shh, we obtained an anti-Shh hybridoma supernatant from DSHB and tested it in explant cultures to determine the concentration required to block motoneuron generation in explant cultures. A concentration of 50 μl/ml of supernatant was sufficient to completely block the generation of p75-immunoreactive motoneurons

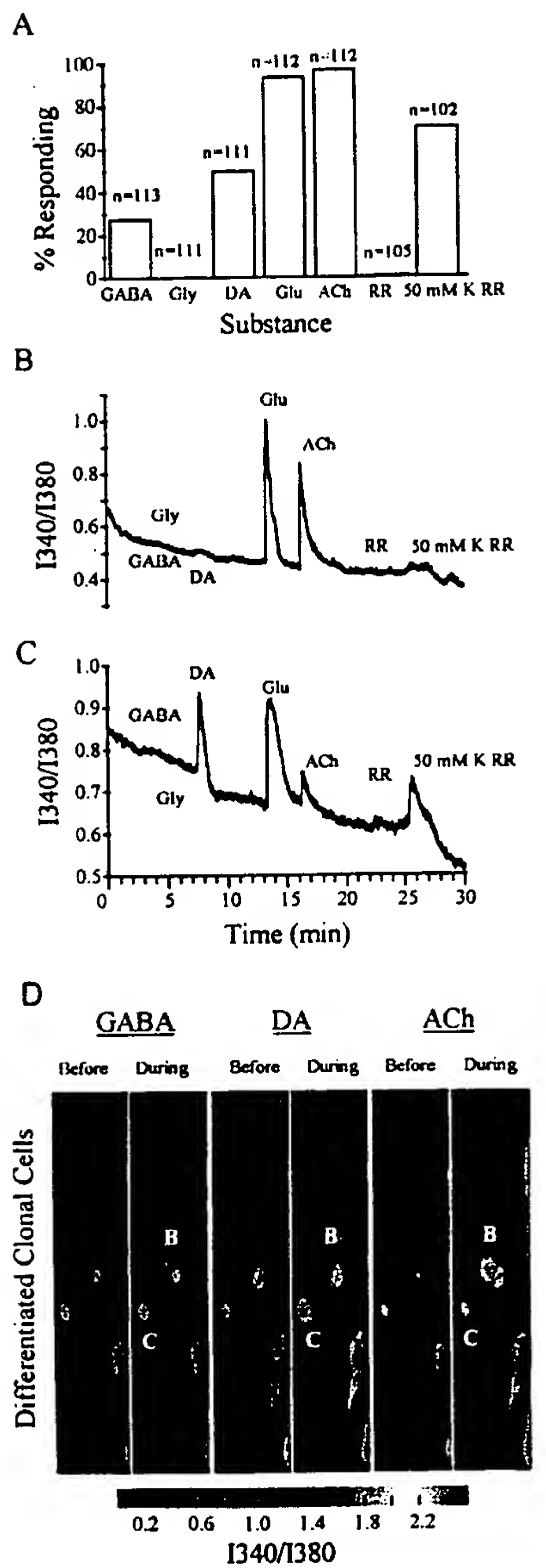

Figure 5. Individual E-NCAM$^+$ cells respond to multiple neurotransmitters. Ca^{2+} imaging using fura-2 showed that clonal cells generated from individual E-NCAM$^+$ neuroblasts were capable of expressing different neurotransmitter receptors. *A*, The *bar graph* shows the percentage of differentiated clonal cells that responded to 500 μM GABA, glycine (*Gly*), dopamine (*DA*), glutamate (*Glu*), acetylcholine (*ACh*), rat Ringer's control (*RR*), and 50 mM K^+. *B, C*, The ratio of intensity from excitation at 340 nm and 380 nm plotted over time shows relative changes in $[Ca^{2+}]_i$ from two cells differentiated from one clone in response to neurotransmitters RR control and 50 mM K^+. *D*, Shown are images of differentiated cells from one clone before and during responses to neurotransmitters RR control and 50 mM K^+. The *letters* indicate the cells used for the traces in *B* and *C*.

(data not shown). The same concentration of Shh neutralizing antibody also completely blocked the effect of Shh on mitosis. In contrast, a control hybridoma supernatant had no inhibitory effect on Shh-induced mitosis (Fig. 7*A*).

To demonstrate that the observed effects of Shh on mitosis were caused by signaling through the patched–smoothened receptor complex, we examined expression of *patched* (*ptc*) and *smoothened* (*smo*) in dissociated E-NCAM$^+$ cells. As can be seen in Figure 7*B*, both patched and smoothened were readily detected, indicating that the receptors are expressed by E-NCAM$^+$ cells. Patched protein is thought to be downregulated after binding Shh, whereas *ptc* message is upregulated. We therefore compared the expression levels of *ptc* mRNA from Shh and control treated as E-NCAM$^+$ cells. E-NCAM$^+$ cells were grown alone, or in the presence of Shh (as described Materials and Methods) for 5 d and were harvested for RNA isolation and cDNA synthesis. Relative levels of *ptc* expression from each population were estimated and compared by using *cyclophilin/ptc* ratios (as described in Materials and Methods). Results from three independent experiments indicate that Shh significantly upregulates the message for *ptc* (1.48 ± 0.18 SEM; control = 1.00). Thus, Shh directly acts on E-NCAM$^+$ cells to regulate cell proliferation via activation of the Patched signaling pathway.

Although Shh had an obvious effect on mitosis, it had no apparent effect on p75 or ChAT expression in dissociated cultures of E-NCAM$^+$ cells (Fig. 6*E,F*). No cholinergic differentiation was apparent at any dose of Shh tested, and differentiation of glutaminergic or GABAergic phenotypes was not observed (data not shown). The failure to see cholinergic neurons was not caused by an inability of the E-NCAM$^+$ cells to differentiate into p75- or ChAT-positive cells, because sister cultures readily differentiated into ChAT- and p75-immunoreactive cells when treated with a differentiation agent such as BMP-2 (Fig. 6*F,I*) or RA (data not shown). Thus, E-NCAM$^+$ cells in dissociated culture respond to Shh by increasing cell division and by an inhibition of differentiation, in contrast to the response observed in explant cultures (our results) (Ericson et al., 1996).

Our results indicate that the extracellular signaling molecules Shh and BMP-2 modulate the phenotypic differentiation of E-NCAM$^+$ cells. BMP-2 inhibits cell proliferation and promotes differentiation and does not inhibit the differentiation of ventral phenotypes. In contrast, Shh promotes proliferation and inhibits the differentiation of any neuronal phenotypes, including p75- and ChAT-immunoreactive neurons.

DISCUSSION

The data presented here demonstrate that individual, dividing E-NCAM$^+$ NRPs have the potential to generate multiple types of mature neurons that synthesize and respond to multiple neurotransmitters, including dopamine, acetylcholine, GABA, and glycine. NRP cells progress toward maturity with changes in mitotic activity, neurotransmitter response profile, and the expression of cell-type and stage-specific markers, such as neurotransmitters or their synthetic enzymes, synaptophysin, and NF-M. Finally, we report that differentiation of NRP cells can be modulated by Shh and BMP-2.

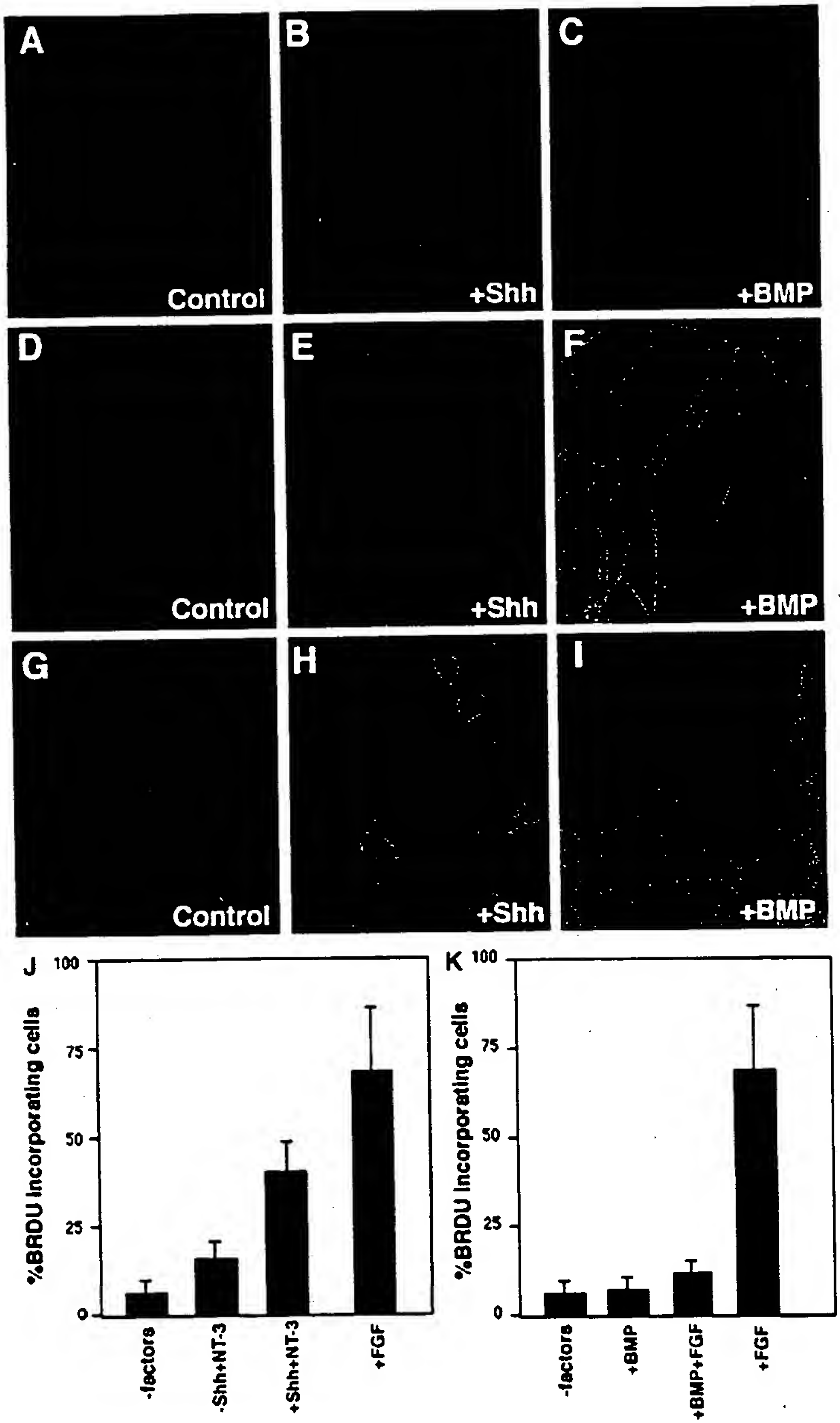

Figure 6. Shh acts on E-NCAM⁻ neuroblasts to promote cell division, whereas BMP-2 acts on E-NCAM⁻ neuroblasts to promote differentiation. E-NCAM⁻ cells were isolated and grown in NEP basal medium supplemented with FGF and NT-3 (*A, D, G*) with the addition of Shh (100 ng/ml) (*B, E, H*) or BMP-2 (10 ng/ml) (*C, F, I*). After 5 d in culture (48 hr for BMP-2), cultures were fixed, and cell division (*A, B, C*), p75 immunoreactivity (*D, E, F*), and ChAT expression (*G, H, I*) were compared. Comparison of cell division by BRDU incorporation showed that Shh promoted proliferation, whereas BMP-2 inhibited proliferation (compare *A, B*, and *C*). In contrast to the effect of Shh on proliferation, no detectable p75- or ChAT-immunoreactive cells (*E* and *H*, respectively) were seen after Shh treatment for 5 d. In contrast, BMP-2 treatment for 48 hr allowed differentiation of large numbers of p75- and ChAT-immunoreactive cells (*F* and *I*, respectively). *J* and *K* quantify the effect of Shh and BMP-2 on cell division. Cells were pulse-labeled with BRDU for the last 24 hr in culture, and the number of BRDU-incorporating cells was counted. *Shh+NT-3* caused a threefold increase in BRDU incorporation when compared with control conditions in which cells were treated with NT-3 alone. The effect of Shh was not as dramatic as that of FGF. In contrast, BMP-2 caused a reduction in BRDU incorporation, and this effect of BMP-2 overrode the mitogenic effect of FGF. Objective magnification, 20×.

A major finding in this study is that neuronal precursor cells can generate heterogeneous progeny: clones of mature neurons that synthesize multiple neurotransmitters and differentially respond to them. More importantly, in all clones analyzed, single precursor cells generated a heterogeneous population of mature cells as assessed by immunocytochemistry and response to neurotransmitter application. The proportion of cells that responded to any single neurotransmitter varied from clone to clone, but in

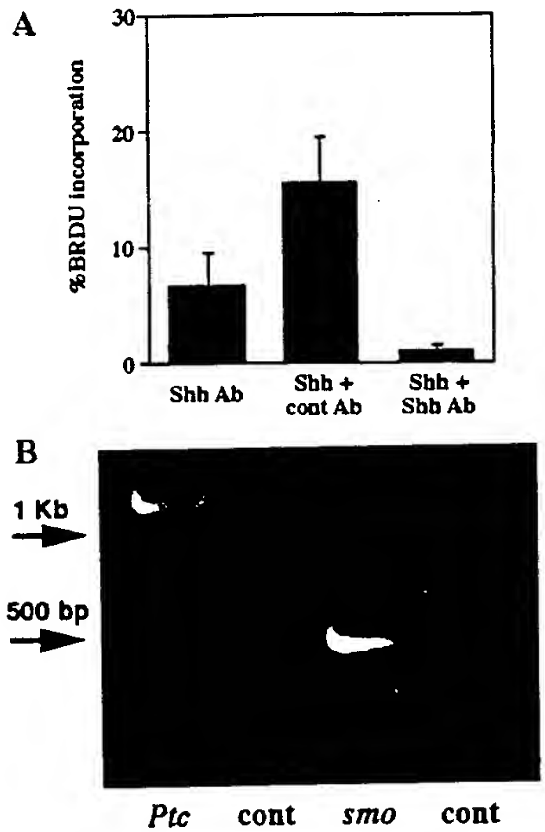

Figure 7. The mitotic effect of Shh can be blocked by neutralizing antibodies directed against Shh. E-NCAM$^+$ cells were isolated and grown in NT-3-supplemented NEP basal medium with the addition of neutralizing anti-Shh hybridoma supernatant (50 μl/ml), Shh (100 ng/ml) with a control hybridoma supernatant, or Shh (100 ng/ml) plus neutralizing anti-Shh hybridoma supernatant. Cells were grown for 48 hr and pulsed with BRDU for the last 3 hr of culture, and the number of BRDU-incorporating cells was counted. The mitotic effect of Shh on dissociated E-NCAM$^+$ cells was completely blocked in the presence of the neutralizing Shh antibody. In contrast, no effect was seen with a control (anti-A_2B_5) hybridoma (*A*, mean of 2 independent experiments). Sister plates of E-NCAM$^+$ cells were grown in culture for 48 hr, harvested, and analyzed for *ptc* and *smo* expression by RT-PCR. Both *ptc* and *smo* were readily detected (*B*; $n = 2$).

all cases, cells that responded to either GABA, glutamate, glycine, acetylcholine, dopamine, or some combination could be identified, indicating that multiple neuronal phenotypes can arise from a single neuronal precursor. Additionally, although the NRPs were taken from the developing spinal cord, they were not limited in their potential to spinal cord cells, because we detected a significant number of dopamine-synthesizing cells that are normally not present in the spinal cord. Our clonal data also suggest that at this stage, neuronal precursors are not committed to generating only dorsal or only ventral phenotypes, because we detected cholinergic neurons as well as other neuronal phenotypes together within the same clone. Consistent with our results are results from earlier retroviral studies showing that clones in chick spinal cord can generate motoneurons as well as other neurons even relatively late in their development (Leber et al., 1990; Leber and Sanes, 1995). Mass culture assays such as those described by Ray and Gage (1994) and Richards et al. (1995) provide additional confirmation for our observations. Taken together, these results suggest that in the spinal cord, progenitor cells have made a commitment to become neurons before becoming postmitotic and acquiring a specific neuronal phenotype.

The transition of E-NCAM$^+$ cells from immature NRPs to mature, functional neurons occurs in stages, aspects of which we have identified (summarized in Table 1). Our results suggest that immature dividing precursors express E-NCAM and MAP-2 kinase, many express β-III tubulin, and only a small subset of the cells respond to excitatory neurotransmitters. These results are consistent with the expression pattern of NF and β-III tubulin described in the developing spinal cord (Chen and Chiu, 1992), as well as the development of electrical activity described in mixed cultures [Walton et al., 1993 (for review, see Spitzer, 1991)]. Of importance to note was our observation that responses to GABA and glycine by immature cells were depolarizing. Although depolarizing responses are not surprising for ACh, dopamine, and glutamate application, the depolarizing responses to GABA and glycine are different from what might be expected for transmitters that play an inhibitory role in the mature nervous system. Nevertheless, similar depolarizing responses to both GABA and glycine have been described in embryonic spinal cord cultures (Fulton et al., 1980; Takahashi, 1984; Jahr and Yoshioka, 1986; for review see Cherubini et al., 1991). Depolarizing responses generally undergo reversal in cultures from early postnatal animals (Ben-Ari et al., 1989). We noted that mature differentiated E-NCAM$^+$ cells did not show a depolarizing response to GABA or glycine, despite the expression of their receptors, suggesting that a similar maturation had occurred in culture. These results suggest that E-NCAM$^+$ cells mature in culture and that this maturation mirrors many aspects of the maturation described for *in vivo* development of the spinal cord. The ability to analyze stages of normal development in purified populations of cells will allow for a more detailed dissection of the coordinate acquisition of maturation markers.

Our present results show that BMP-2 acts to promote neuronal differentiation from neuronal-restricted precursors. This effect is likely stage specific and direct (i.e., BMP-2 is not inducing the release of other factors from non-neuronal cells) because we used purified populations of NRP cells. We have shown previously that BMP-2 acts on NEP cells, the immediate precursors of NRP cells, to generate neural crest derivatives (Mujtaba et al., 1998). Furthermore, we and other groups have shown that BMP-2 can act on glial precursors to promote astrocytic differentiation (D'Alessandro et al., 1994; Gross et al., 1996; Mabie et al., 1997; and our unpublished results). These results illustrate the importance of examining the role of individual factors on purified cell populations at specific stages in development. The specific effect of BMP-2 on cultured cells will depend on the age of the cultured cells and on which precursor cells are present in a mixed population of cells. The specific mechanism (or mechanisms) that underlies the differential response of a precursor to BMP-2 remains to be determined.

Previous studies have shown that several members of the BMP family, including BMP-4, can promote dorsal phenotypes in explant cultures of chick spinal cord (Liem et al., 1995, 1997). Our results show that in dissociated culture there appears to be no inhibition of ventral phenotypes or a bias toward dorsal phenotypes. Rather, BMP-2/4 appeared to nonspecifically promote differentiation. These results are consistent with dorsal notochord transplant experiments (Placzek et al., 1990; Yamada et al., 1991, 1993), and ectopic Shh application experiments (Echelard et al., 1993; Roelink et al., 1994) *in vivo* showing that even in the

presence of endogenous BMP-2, ventral phenotypes are readily generated, suggesting that BMP-2 does not inhibit ventralization *in vivo*.

Our results analyzing the effect of Shh on E-NCAM$^+$ precursor cells indicate that it acts primarily as a mitogen and does not promote cholinergic neuronal differentiation at this stage of development. The mitotic effect of Shh was not unexpected, because Shh has been shown to be a mitogen in various assays. For example, Shh has been shown to promote the proliferation of retinal precursor cells (Jensen and Wallace, 1997) and mouse sclerotome cells (Fan et al., 1995). Similarly, application of Shh to CNS stem-like cells (Flax et al., 1997), as well as to human lung squamous carcinoma cells (Fujita et al., 1997), also stimulates cell proliferation. Our results suggest that, as in other regions, one function of Shh in the spinal cord may be to promote cell division. However, our failure to see motoneuron differentiation was somewhat unexpected, because motoneuron differentiation in response to Shh application has been shown in explant cultures (Echelard et al., 1993; Roelink et al., 1994). Absence of motoneuron differentiation is not caused by an absence of the receptor, because both *patched* and *smoothened* are expressed by these cells. It also appears unlikely that an insufficient concentration of Shh was used, because even doses as high as 500 ng/ml did not show any motoneuron differentiation (see Results) (our unpublished observations). Much lower doses of Shh than the 100 ng/ml used here will readily promote generation of Islet-1/ChAT-immunoreactive motoneurons in explant cultures over the same time period (Echelard et al., 1993; Roelink et al., 1994). The absence of cholinergic differentiation in our cultures cannot be attributed to the time period of the assay, because we did not see any appreciable motoneuron differentiation even after prolonged exposure to Shh. Furthermore, E-NCAM$^+$ cells readily differentiated into motoneurons 48 hr after exposure to RA or BMP-2, indicating that the failure to see motoneuron differentiation is likely attributable to the mitogenic effect of Shh.

Previous studies suggest possible explanations for our results. Two periods of Shh signaling have been shown to be required for motoneuron specification (Ericson et al., 1996): an early period, during which Shh may convert NEP cells into ventralized precursors, and a late period, during which Shh may drive the differentiation of ventralized progenitors into motoneurons. We may be seeing the effect of only the early period of Shh application. Alternatively, it has recently been shown that vitronectin is required for motoneuron generation in the presence of Shh, but that vitronectin is not required for the patterning effects of Shh (Martinez-Morales et al., 1997). Perhaps sufficient vitronectin is not present in our relatively low-density cultures. Further experiments will be necessary to clarify our finding that Shh did not promote motoneuron differentiation in dissociated cultures. Nevertheless, our results clearly show that Shh acts at the stage of neuronal precursors to regulate their proliferation, and that E-NCAM$^+$ cultures may provide a ready assay to dissect out the role of Shh in regulating motoneuron differentiation.

In summary, we have shown that E-NCAM immunoreactivity identifies a self-renewing, NRP cell that can generate multiple neuronal phenotypes, revealing that lineage commitment to the neuronal cell type precedes its specific phenotypic commitment. In addition, this cell is amenable to perturbation by extracellular factors, allowing the processes of lineage restriction and neuronal differentiation to be characterized in detail.

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Low Ca^{2+} Stripping of Differentiating Cell Layers in Human Epidermal Cultures: An *in Vitro* Model of Epidermal Regeneration

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An *in vitro* model of the epidermal regeneration process is described. Incubation of multilayered, keratinizing cultures of human epidermal cells in Ca^{2+}-free medium for 72 h results in a complete stripping of all suprabasal layers. When the Ca^{2+} stripped cultures are refed normal Ca^{2+} medium a reproducible series of morphologic and cell kinetic changes takes place. It is suggested that these changes represent a general pattern of regeneration after epidermal wounding. After an initial lag phase the regenerative response is principally effected by a recruitment to the proliferating pool of cells with a high rate of DNA synthesis. The cells seem to be programed to rapid differentiation. Studies with cholera toxin suggest that this adenylate cyclase-stimulating agent is able to induce significant changes in the regenerative process causing a prolonged, but less intense, proliferative response leading to lateral growth rather than to rapid differentiation.

In previous reports we have shown that proliferating cells in human keratinocyte cultures can be divided into at least two subpopulations on the basis of their rate of thymidine incorporation during S-phase [1, 2]. Studies with growth stimulators and inhibitors have suggested that these subpopulations may be differentially involved in regneration and early differentiation of the keratinocyte. [3, 4]

Epidermal wounding is followed by a regenerative response consisting of a burst of proliferative activity seen about 3 days after the tissue damage [5–11]. Evidence has been presented to show that basal cell subpopulations may respond differently and selectively during the wound healing process [12–15].

In the present report we describe an *in vitro* equivalent to epidermal regeneration. It is suggested that stripping of the uppermost, differentiating layers in human epidermal cultures induces the selective proliferation of a cycling subpopulation with a high rate of DNA replication and a commitment to rapid differentiation. In the presence of cholera toxin this regenerative response is modified.

MATERIALS AND METHODS

Cell culture. The method of obtaining pure cultures of human epidermal keratinocytes has been described in detail elsewhere [1, 16]. Normal adult whole skin was sampled from plastic surgery operations. Before cultivation as much as possible of the subepidermal tissue was removed by gentle

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scraping. Primary cultures were initiated by an explant method with eight explants per 25-cm² tissue culture flask. The cultures were grown in Dulbecco's Modified Eagle medium (DMEM, GIBCO) plus 1.68 g/liter $NaHCO_3$, 20% (v/v) fetal calf serum (GIBCO), penicillin 250 U/ml, and streptomycin 25 μg/ml. The medium was further supplemented with hydrocortisone 0.4 μg/ml and epidermal growth factor (EGF, Collaborative Research Inc., Waltham, MA) 10 ng/ml. The cultures were equilibrated with 5% CO_2 in air and incubated at 34–35°C. Medium was changed twice a week.

Stripping with low Ca^{2+} medium. One-month-old primary cultures were used for all experiments. At this stage most culture flasks were subconfluent and extensive keratinization with desquamation prevailed. Experience with epidermal cell cultures grown in medium with reduced Ca^{2+} concentration has shown that Ca^{2+} is essential for cell–cell cohesion [17–19]. When epidermal cultures are maintained in low Ca^{2+} medium for extended periods the rate of desquamation of the differentiating cells is greatly increased [18, 20]. In the present study stripping of the differentiating cell layers was performed by incubating the cultures in Ca^{2+}-free minimal essential medium (GIBCO) without additives at 37°C in an atmosphere of 5% CO_2 in air. After 72 h all suprabasal layers were detached as differently sized flakes that could easily be removed after gently shaking of the culture flask. The cultures were then refed medium with normal Ca^{2+} concentration as described above in order to initiate the regenerative reaction (time = 0). The culture morphology and mitotic activity were followed daily for the next 7 days in the phase-contrast microscope and photographs were taken at different times.

In some experiments the low Ca^{2+} stripped cultures were refed normal Ca^{2+} medium supplemented with 10^{-9} *M* cholera toxin (CT, Sigma Chemical Co.) and with elevated concentration of EGF (20 ng/ml compared to 10 ng/ml in the "normal" medium described above). This medium in earlier studies has been shown to stimulate lateral growth and inhibit differentiation in primary explant cultures of human epidermal keratinocytes [3].

[³H]Thymidine labeling, cell sorting, and autoradiography. At appropriate times after being stripped cultures were labeled with [³H]thymidine ([³H]dThd) for combined analysis by cell sorting and autoradiography. All labeling experiments were performed with [³H]dThd (New England Nuclear, sp act 50–80 Ci/mmol) at a concentration of 10 μCi/ml for 30 min at 37°C [2]. After labeling, the cells were prepared for cell sorting and autoradiography as previously described in detail [1, 2]. A single cell suspension was obtained by trypsinization and the cells were fixed in absolute ethanol. The cells were then stained with a combination of mithramycin and ethidium bromide for flow cytometry of cellular DNA contents. The DNA frequency distributions were obtained with a fluorescence-activated cell sorter (FACS II) (Beckton–Dickinson, CA). Cells were sorted from a window of 10 channels in the middle of the S-phase DNA distribution (spanning over 30 channels) and from a window in the G_1-phase of the cell cycle representing the left (lower) half of the G_1 peak. The sorted cells were collected on glass slides in a drop of ethanol, dried, and covered with an Ilford K2 emulsion for autoradiography. The autoradiograms were exposed for 8 days, developed, and finally stained with Giemsa. The number of labeled and unlabeled S-phase cells were counted in the light microscope (magnification ×10³). Three to five hundred cells were counted in each experimental group. The labeled cells were further characterized by counting the number of grains per labeled nucleus to obtain a grain count distribution [1, 2]. The lower limit for accepting a cell as labeled was based on the autoradiographic background (generally ≤ three grains per nucleus). In all experiments more than 98% of cells sorted from the G_1-peak were classified as unlabeled.

The proportion of cells in the different phases of the cell cycle was obtained by planimetry of the DNA frequency distributions [21], with the simple assumptions that the G_1 population is twice the lower half of the G_1 peak, the G_2 population is twice the upper half of the G_2 peak, and the S-phase population is represented by the square given by the distance between the G_1 and G_2 peaks times the height of the mid-S-phase part of the DNA histogram.

The cell diameter of unlabeled S-phase cells collected on glass slides after cell sorting was determined with the aid of a measure introduced into the eyepiece of the microscope. The measure was calibrated using a micrometer slide. All measurements were performed at ×700 magnification and 50–150 cells were analyzed on each slide.

RESULTS

Stripping in Low Ca^{2+} Medium

One-month-old primary cultures were subjected to different treatments to strip off the suprabasal cell layers. Before stripping the cultures covered 75% or more of the culture surface and showed extensive multilayering and keratinization (Fig. 1*A*). Stripping was initially attempted by incubating in NH_4Cl and β-mercaptoethanol according to the method described by Tong and Marcelo [22]; however this treatment apparently caused irreversible damage to the cells as they were unable to reestablish growth and differentiation after being refed with normal medium. In contrast, when cultures were incubated in Ca^{2+}-free medium for 72 h this resulted in a reproducible and selective stripping of all suprabasal layers leaving a monolayer of basal-like cells. Occasional areas of total denudation were seen in some culture flasks, but at least half of the culture surface was always covered by the monolayer. When incubation was performed for only 48 h this resulted in incomplete stripping leaving scattered islets with a multilayered morphology.

Morphological Changes after Refeeding with Normal Ca^{2+} Medium

When the cultures were refed with normal Ca^{2+} medium a reproducible series of morphological changes took place. During the first 24 h the cultures consisted of a monolayer of basal-like cells that appeared somewhat retracted with wide intercellular spaces (Figs. 1*B*, 1*C*). No mitotic activity was observed. By 24 h the cultures started to become heteromorphic. There appeared a few scattered multilayered areas of closely packed cells, whereas the rest of the culture was still occupied by a monolayer of retracted cells. Mitotic figures were occasionally observed (Fig. 1*D*). During the next 2 to 3 days a burst of mitotic activity occurred which peaked at 72 to 96 h after stripping. At this time mitoses occurred all over the culture and not just at the periphery of individual outgrowth zones which is the usual finding under conditions of rapid lateral expansion in young explant cultures. In the stripped cultures no sign of lateral growth was seen during the observation period, but concomitant with the proliferative burst a progressive differentiation and multilayering was observed. At 96 h poststripping the first signs of keratinization appeared (Figs. 1*E*, 1*F*, 1*G*). There was a gradual decrease in mitotic activity, as the keratinization proceeded. Seven days after stripping keratinization and desquamation was extensive and the culture morphology was quite similar to that observed immediately before stripping (Fig. 1*H*).

Flow Cytometry and Labeling of Sorted S-Phase Cells

The relative proportion of cells in the various phases of the cell cycle was measured from the DNA histograms by planimetry. The cells were detached with trypsin, fixed, and stained with a combination of ethidium bromide and mithramycin for flow cytometric measurement of DNA contents. The variation in proportion of G_1- and S-phase cells with time after stripping is shown in Figs. 2*A* and 2*B*. The curve for G_2-phase cells was similar to that for S-phase cells (data not shown). It appears that between 2 and 4 days poststripping there is a decrease in the proportion of G_1-phase cells and a corresponding rise in the S- and G_2-phase fraction. This is coincident with the time of maximal mitotic activity.

All cultures were pulse labeled with [³H]dThd for 30 min prior to harvest and the cells were subjected to sorting and autoradiography. The variation in the grain

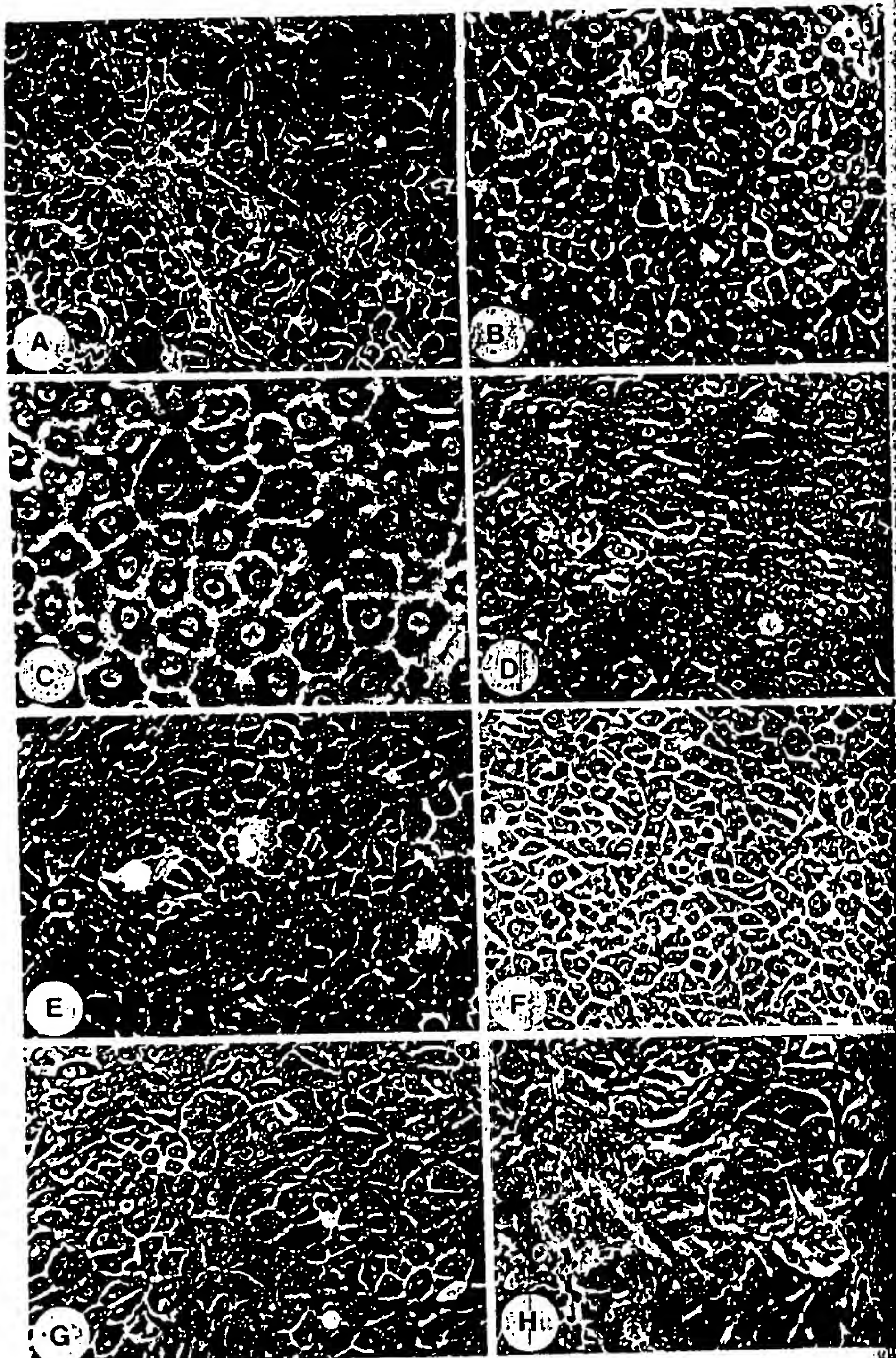

Fig. 1. Morphological changes after refeeding normal Ca^{2+} medium to low Ca^{2+} stripped hum[an] epidermal cultures. (*A*) Immediately before stripping. (*B*) 2 h. (*C*) 8 h. (*D*) 24 h. (*E*) 48 h. (*F*) 72 h. (*G*) [4 days?] b, and (*H*) 7 days poststripping. Note extensive keratinization in (*A*) and (*H*). In (*B*) and (*C*) the culture consists of a monolayer of basal cells. Note the large variation in cell size among basal cells in (*C*). From (*D*) to (*G*) a gradual increase in the number of cell layers is apparent. In (*E*) to (*G*) large numbers of mitotic figures are observed (arrows). Magnification ×80, in (*C*) ×160.

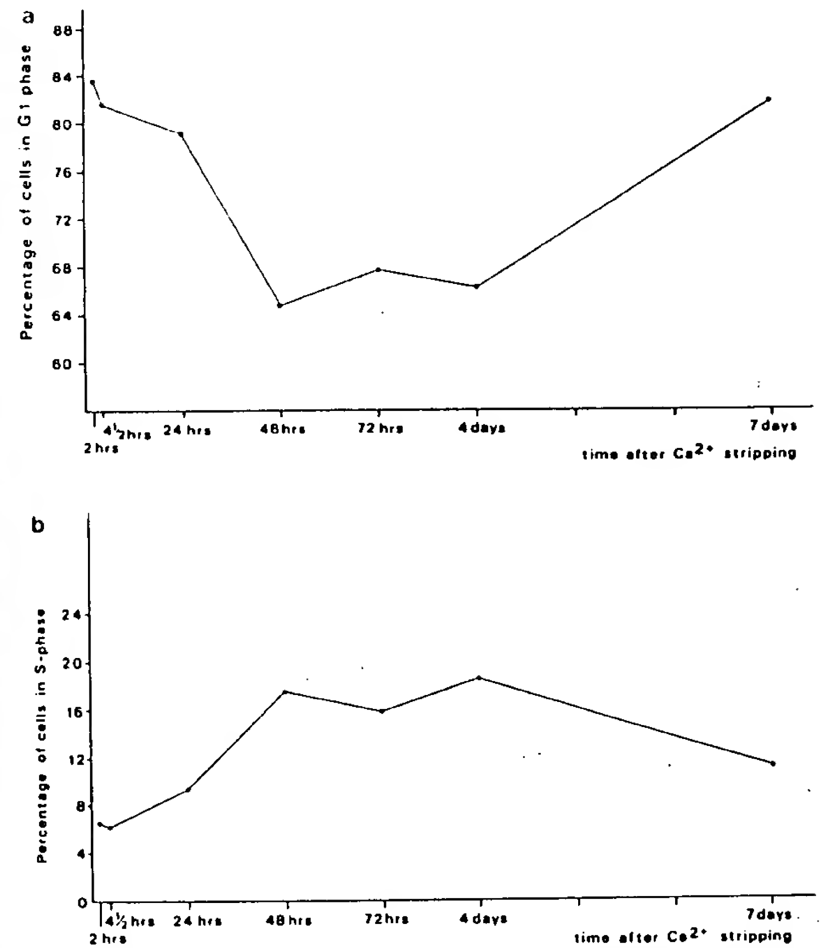

Fig. 2. Variation in the percentage of cells in G_1-phase (*A*) and S-phase (*B*) with time after stripp[ing]. The values represent the means of three separate experiments.

count distribution of sorted S-phase cells with time after stripping is shown for one representative experiment in Fig. 3. The S-phase cells have been divided into three subpopulations with respect to the number of autoradiographic grains [1, 2]: unlabeled (0–3 grains per nucleus), weakly labeled (4–24 grains per nucleus), and strongly labeled cells (more than 25 grains per nucleus). Immediately poststripping the proportion of strongly labeled cells is relatively high and then drops to a minimum 4 1/2 h after stripping. The proportion of strongly labeled S-phase cells then rises dramatically to a maximum of about 75% 3 to 4 days poststripping coincident with the time of rapid proliferation. This is followed by a decrease to

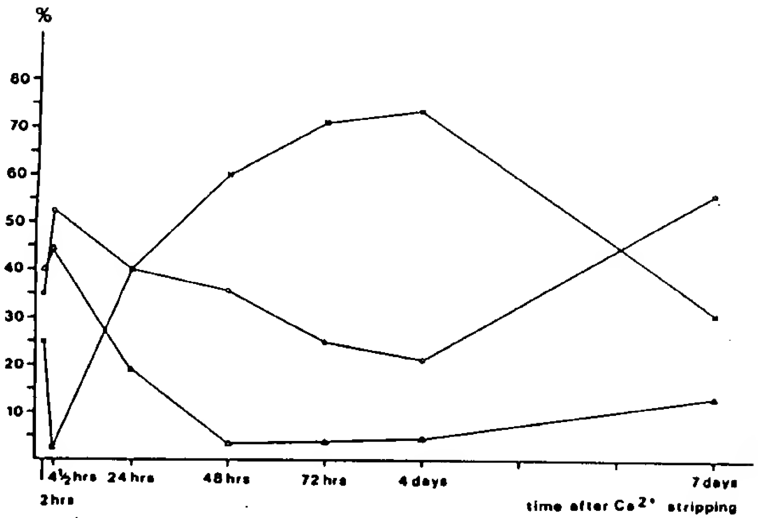

Fig. 3. Variation in the grain count distribution among sorted S-phase cells with time after stripping. The cells were classified into three groups according to the number of nuclear grains: (Δ) 0–3 grains/nucleus. (O) 4–24 grains/nucleus. (×) ≥25 grains/nucleus. Note the relatively strong labeling after 2 h compared to 4 1/2 h poststripping.

about the original level. The fraction of unlabeled S-phase cells drops to a minimum within 2 days and then increases slowly again after the fourth day poststripping.

The Effect of Cholera Toxin on the Regenerative Response

The addition of CT to the culture medium after low Ca^{2+} stripping caused a marked alteration in the regenerative response. Morphologically, the onset of multilayering and visible keratinization was retarded. At 7 days, when cultures in normal medium were strongly keratinizing, keratinization was hardly visible in cultures grown in the presence of CT (Figs. 4*A* and 4*B*). Further evidence for retarded differentiation in the presence of CT was sought from cell size measurements since the size and the state of differentiation of the keratinocyte has been shown to be positively correlated both *in vivo* and *in vitro* [23, 24]. The mean cell diameter of unlabeled S-phase cells was determined at different time points after stripping. In cultures refed medium without CT a significant ($P<0.001$) increase in size of these cells was observed with time whereas no increase could be seen in cultures grown in medium with CT (Table 1).

The increase in the proportion of cells in S-phase and the increase in the proportion of S-phase cells that were strongly labeled with [^{3}H]dThd were also retarded in stripped cultures grown in the presence of CT (Table 1). Whereas these cell kinetic parameters showed a maximum 3–4 days poststripping and then decreased in cultures without CT (Figs. 2 and 3) no distinct maximum was observed in cultures with CT; instead, after a prolonged lag phase a steady increase in both parameters was observed during the observation period. At 7

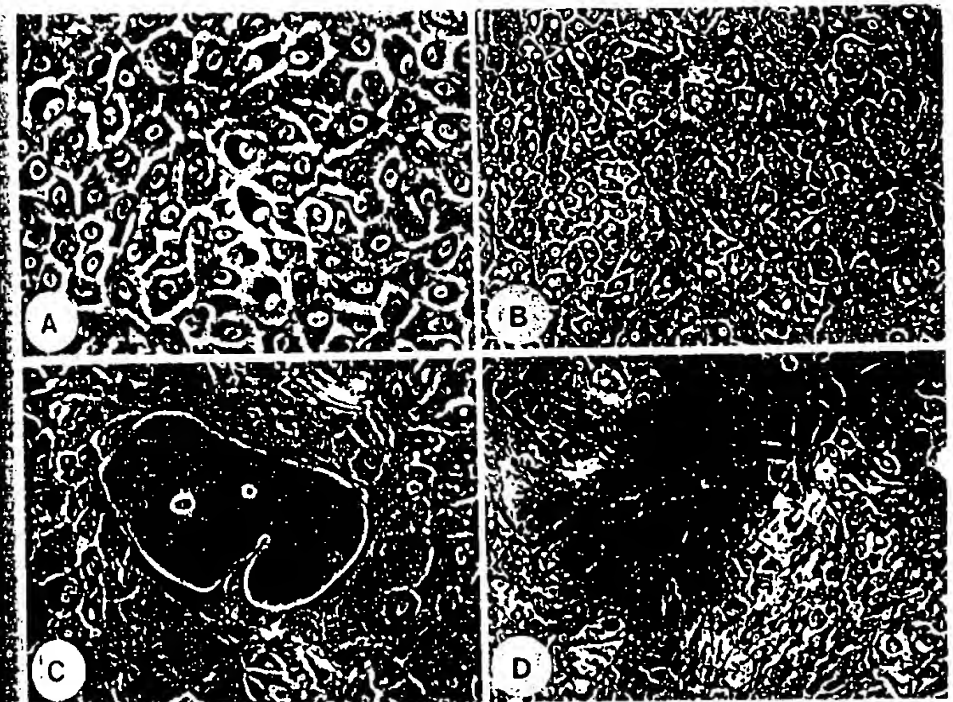

Fig. 4. Morphologic appearance of low Ca^{2+} stripped cultures after addition of normal Ca^{2+} medium supplemented with 10^{-9} *M* cholera toxin and 20 ng/ml EGF. (*A*) Immediately poststripping. Note many cells with dark cytoplasm. (*B*) 9 days poststripping. Note absence of keratinization. (*C*) and (*D*) 7 days poststripping. In (*C*) an initially large denuded area is represented by a remaining small hole in the culture, whereas in (*D*) the denuded area has been completely covered with laterally growing cells. Arrows mark boundary of the most recently covered area. Note mitotic figures within and around that area. Magnification ×80, in (*A*) ×160.

days the proportion of cells in S-phase and their degree of [^{3}H]dThd labeling were much higher in cultures with CT than in control culture. Moreover, in the presence of CT, clear signs of lateral growth were observed from the fourth day on, and many denuded areas were almost completely covered by a monolayer of epithelial cells by Day 7 (Figs. 4*C* and 4*D*). In contrast, no lateral growth was observed in experiments with medium without CT; however, when the observation period was extended beyond Day 7, some lateral growth was also observed in cultures without CT (data not shown).

DISCUSSION

During wound healing, reepithelialization is a process essential to the restoration of the integrity of the integument. The data presented in the present report show that incubation in low Ca^{2+} medium reproducibly detaches the suprabasal layers in human epidermal cultures. The observed cell kinetic and morphologic changes after stripping are assumed to reflect the general regenerative response to injury of the epidermis.

TABLE I

Characteristics of S-phase cells in epidermal cultures maintained in medium with or without cholera toxin for different periods after stripping in low Ca^{2+} medium

Time after low Ca^{2+} stripping	% of cells in S-phase		% of S-phase cells strongly labelled		Mean cell diameter (μm) of unlabelled S-phase cells	
	Control	+CT	Control	+CT	Control[a]	+CT[a]
4 1/2 h	6.2	6.1	2.5	8.4	14.2±3.0[b]	14.8±3.1
24 h	9.3	7.3	40.4	5.9	14.6±3.9	14.5±3.5
7 days	11.0	15.2	31.0	60.1	16.1±2.9[b]	14.6±3.8

Note. Control, grown in DMEM supplemented with 20% fetal calf serum and 10 ng/ml EGF. +CT, grown in DMEM supplemented with 20% fetal calf serum, 20 ng/ml EGF, and 10^{-9} *M* cholera toxin.
[a] Mean ±SD.
[b] Significantly different from each other ($P \leq 0.001$).

Normally, the desquamating keratinized cells of the epidermis are replaced through mitotic division of the cells of the basal layer. In the normal steady state the rate of loss of terminally differentiated surface cells by desquamation is balanced by a controlled rate of cell production.

The proliferative reaction of the epidermis to acute tissue injury has been studied in detail in both human and animal skin *in vivo* [5–7, 10, 15, 23, 24] and *in vitro* [8, 27]. It consists of an initial lag phase of depressed cell proliferation followed by a dramatic, but transient, burst of mitotic activity about 3 days after the injury. During the phase of rapid proliferation the *mean* cell cycle time is strongly reduced [9, 15]. Concomitant with the proliferative response characteristic morphological and immunological changes are observed [28–30].

Experimental wounding has mostly been accomplished by adhesive tape stripping which removes a variable number of differentiating layers of the epidermis [5, 6, 8, 9, 11, 25]. Others have used cutting [6, 8] or application of skin irritants [26]. These studies have confirmed the general pattern of the regenerative response occurring independently of the nature of the external stimulus. Moreover, the strength of the regenerative reaction is directly related to the strength of the stimulus (i.e., extent of loss of differentiating cells) [6, 8].

The present study seems to confirm the general pattern of regenerative proliferation after epidermal wounding. An initial lag phase of less than 24 h was followed by a burst of mitotic activity which peaked around the third to fourth day after stripping. During the lag phase the cultures consisted of a monolayer of basal-like cells without visible mitotic activity. The labeling index of the sorted S-phase cells was only about 50% (Fig. 3) suggesting an inhibition of DNA replication. During the following days the parallel increase in proliferative activity and multilayering was accompanied by an overall increase in the S-phase labeling index to nearly 100% (Fig. 3). Simultaneously, a dramatic increase in the proportion of strongly labeled S-phase cells appeared (Fig. 3). During the peak of proliferative activity the strongly labeled S-phase cells amounted to about 75% of S-phase cells, whereas under conditions of rapid lateral growth in young

explant cultures they constitute only about 40% [2]. Furthermore, as seen in Fig. 2 the proportion of S-phase cells was increased at the expense of G_1-phase cells during the peak of proliferate activity. These findings agree with observations made after injury to mouse epidermis *in vivo* [25, 31]. The results taken together indicate that the regenerative response principally was effected by a recruitment to the proliferating pool of cells with a high rate of DNA replication. The cells seemed to be programed to rapid differentiation (perhaps after only one cell cycle) and not to continued proliferation as no sign of lateral growth was observed. Furthermore, the phase of rapid proliferation was followed by a phase with increasing keratinization and, from the sixth or seventh day, an increasing desquamation. During this phase the grain count distribution among sorted S-phase cells was gradually returned to a steady-state pattern [2], which was reached at the seventh day. From these data a possible biological function of the strongly labeled S-phase cells is implicated: They may represent the so-called transitional amplifying cells which are thought to be responsible for expansion of the epidermal cell mass committed to differentiation [32]. This suggestion is compatible with observations made by other investigators studying the epidermal wound healing process [13, 33].

The experiments with CT-containing medium suggest that CT may be able to preferentially enhance lateral growth at the expense of the capacity of the cells to differentiate. This is in agreement with the original work of Green [34] and with our previous observations on primary explant cultures [3] which expand much more rapidly in the presence of CT supposedly due to the increase of the intracellular cAMP concentration [34]. In the present stripped cultures, CT strongly inhibits the rekeratinization and prevents the appearance of larger unlabeled S-phase cells (Table 1). The size of unlabeled S-phase cells was measured since there are several indications that these cells represent both the most undifferentiated, small, basal cells as well as the most differentiated, large, cycling cells; the strongly labeled S-phase cells, on the other hand, constitute an intermediate group of cells of rather uniform size (unpublished observation). The increase in the proportion of strongly labeled S-phase cells is also retarded in the presence of CT. However, this parameter—and the total S-phase fraction—gradually increases (Table 1) concomitant with the initiation of lateral growth. This finding indicates that strongly labeled S-phase cells also represent cells expanding the population for lateral growth.

It has been suggested that the observed heterogeneity in the labeling pattern of S-phase cells in cultured epidermal cells is a technical artifact related to diffusion problems [35]. However, the present observation of parallelism between the peak of multilayering and the peak in the fraction of strongly labeled S-phase cells is a powerful argument against this hypothesis.

It is not yet completely clear why there are such enormous differences in thymidine incorporation among different categories of S-phase cells. One obvious possibility would of course be that the variation is due to fundamental differences in the nucleotide metabolism. However, attempts to abolish the heterogeneity of DNA labeling among S-phase cells by use of other DNA precursors, expansion of

the nucleotide pools, and inhibition of *de novo* nucleotide synthesis have all failed [2]. True differences in the rate of DNA replication among S-phase cells is thus likely to be the major explanation for the observed heterogeneity. More elaborate cell kinetic studies of epidermal systems [36, 37] support the idea of subpopulations of basal cells proceeding with very different rates through the S-phase of the cell cycle.

It is concluded that the regenerative response after low Ca^{2+} stripping of human epidermal cultures is a good *in vitro* model of the epidermal healing process. The overall *in vitro* epidermal regenerative reaction to stripping is very similar to the *in vivo* response after tape stripping. Preliminary experiments (data not shown) show that the stripping can be repeated over and over again on the same culture, opening up the possibility of simulating an aging process *in vitro*. The model thus offers several opportunities to study the developmental relations among the different subpopulations of proliferating cells and their regulation in the epidermis [10, 38–41].

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